Seaweeds as a source of non-digestible complex polysaccharide components for the development of novel prebiotic ingredients for the functional food industry

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Paul Cherry

C D G Em

“You got to__ take good care of business, my friend

C D G

Take good care of yourself

C D G Em

You got to__take good care of business, my friend

C D G

Otherwise you'll end up on the shelf”

Lindisfarne - Taking Care Of Business

Written by Alan Hull

Roll On. Ruby (1973)

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Abstract

This thesis used *in vitro* batch culture fermentation models to investigate the fermentability and prebiotic potential of the brown seaweeds *Fucus vesiculosus* and *Laminaria digitata*, and the red seaweed *Palmaria palmata*. Fermentation substrates included each whole seaweed (a food matrix) and a polysaccharide-rich extract from each seaweed (a mixture of dietary fibre components). Purified seaweed polysaccharides (alginate, fucoidan and laminarin from brown seaweeds; ulvan from green seaweeds; and xylan from red seaweeds) were also evaluated as fermentation substrates to understand the effect of individual seaweed dietary fibre components on gut microbiota composition and metabolic activity. Cellulose and Synergy 1 were used as negative and positive controls, respectively. Analysis was conducted on bacterial composition (qPCR, and 16S rRNA amplicon sequencing) and bacterial metabolic activity (GC-MS to quantify short chain fatty acid (SCFA) concentration).

Fermentation in the presence of the whole seaweed powders of *Fucus vesiculosus* and *Laminaria digitata* resulted in significantly higher concentrations of SCFA when compared cellulose (total SCFA: cellulose = 7214.85 µM; FVWS = 15501.80 µM; LDWS = 23465.80 µM; $p < 0.05$). The whole brown seaweed powders and polysaccharide extracts stimulated glycan degrading bacterial families such as *Bacteroidaceae, Ruminococcaceae* and *Lachnospiraceae* when compared to cellulose. After 24 hrs fermentation, purified alginate polysaccharides yielded significantly higher concentrations of propionic acid (cellulose = 280.64 µM; Alginate-Fv = 725.26 µM; Alginate-Lj = 613.40 µM; $p < 0.05$) and butyric acid (cellulose = 549.43 µM; Alginate-Fv = 944.45 µM; Alginate-Lj = 864.27 µM; $p <$

0.05), respectively. While fermentation in the presence of laminarin also yielded significantly higher concentrations of propionic acid after 24 hrs (cellulose = 280.64 µM; Laminarin-Ld = 933.57 µM; $p < 0.05$). Alginate and laminarin substrates also stimulated populations of *Bacteroides* and *Lachnospiraceae*, respectively, when compared to cellulose.

Fermentation in the presence of the *Palmaria palmata* whole seaweed powder and purified xylan resulted in significantly higher concentrations of SCFA when compared to cellulose (total SCFA: cellulose = 1162.1 µM; Xylan-Pp = 4781.92 µM; $p < 0.05$), whereas the polysaccharide extract did not. Fermentation in the presence of the *Palmaria palmata* whole seaweed powder, polysaccharide-rich extract, and purified xylan elicited a bifidogenic effect.

Future *in vivo* investigations regarding the effect of seaweed polysaccharides on the gut microbiota and host health should investigate the effects of purified alginate, laminarin and xylan, and their respective oligosaccharides. Cost-effective methods to obtain these potential food ingredients are needed.

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Signed…….

PAUL CHERRY

Statement of Collaboration

This research was conducted within the Northern Ireland Centre for Food and Health (NICHE), Ulster University, Coleraine; Teagasc Food Research Centre, Moorepark; National University of Ireland, Galway; Institute of Technology, Sligo; and funded by Funded by The Department of Agriculture Food and the Marine (DAFM) under the National Development Plan 2007-2013, Project number 13F511 (PreMara). I was in receipt of a Department for Employment and Learning Northern Ireland (DEL) Postgraduate studentship which covered university fees and provided a stipend.

The following work was conducted as part of the PhD:

Chapter 1: General introduction

The following work was conducted by me: literature search, appraisal of the literature and the write-up. The critical review of the chapter was carried out by me. Feedback was provided by P Allsopp and E McSorley.

Chapter 2: Literature review

The conception, design, and interpretation were carried out by P Allsopp, E McSorley, P Magee, C O'Hara and I. The literature search, literature appraisal and the write-up were conducted by me, with assistance from C O'Hara. The critical review of the chapter was carried out by me. Feedback was provided by P Allsopp, E McSorley, and P Magee.

Chapter 3: Literature review

The conception, design, and interpretation were carried out by C Stanton, P Allsopp, E McSorley, R P Ross, C Strain, S Yadav and I. The literature search, literature appraisal and the write-up were conducted by me, with assistance from S Yadav. The critical review of the chapter was carried out by me. Feedback was provided by P Allsopp, E McSorley, C Strain, C Stanton and R P Ross.

Chapter 4: Effects of the edible brown seaweeds, *Fucus vesiculosus* and *Laminaria digitata*, on the composition and metabolic activity of the human gut microbiota using an in vitro model of the distal colon

Z Popper, C O' Callaghan, and I harvested the seaweed and Z Popper confirmed the identification of the collected seaweed. The extraction method was carried out by S Yadav and I, based on previously established work by T Smyth and C Strain. The following was solely carried out by me: simulated human digestion of seaweeds and polysaccharide extracts, SCFA analysis by GC-MS, 16S rRNA amplicon sequencing library preparation, qPCR, collection of data, statistical analysis, interpretation of results and write-up. P Allsopp, C Strain, Catherine Stanton and I designed the experiment. The *in vitro* fermentations and bacterial gDNA extractions were carried out by S Yadav and I. 16S rRNA amplicon sequencing was carried out by F Crispie and the bioinformatics analysis by F Fouhy. SCFA analysis via GC-MS was carried out by me, under the supervision of T Smyth. All work relating to the faecal fermentation experiments was carried out at Teagasc Food Research Centre, Moorepark. The critical review of this chapter was conducted by me and feedback was provided by P Allsopp and E McSorley.

Chapter 5: Effects of the edible red seaweed, *Palmaria palmata*, on the composition and metabolic activity of the human gut microbiota using an in vitro model of the distal colon

Z Popper, C O' Callaghan, and I harvested the seaweed and Z Popper confirmed the identification of the collected seaweed. The extraction method was carried out by S Yadav and I, based on previously established work by T Smyth and C Strain. The following was solely carried out by me: simulated human digestion of seaweeds and polysaccharide extracts, 16S rRNA amplicon sequencing library preparation, qPCR, collection of data, statistical analysis, interpretation of results and write-up. P Allsopp, C Strain, Catherine Stanton and I designed the experiment. The *in vitro* fermentations and bacterial gDNA extractions were carried out by S Yadav and I. 16S rRNA amplicon sequencing was carried out by F Crispie and the bioinformatics analysis by F Fouhy. SCFA analysis via GC-MS was carried out by me, under the supervision of T Smyth. All work relating to the faecal fermentation experiments

was carried out at Teagasc Food Research Centre, Moorepark. The critical review of this chapter was conducted by me and feedback was provided by P Allsopp and E McSorley.

Chapter 6: Effects of seaweed polysaccharides on the composition and metabolic activity of the human gut microbiota using an in vitro model of the distal colon

Seaweed polysaccharides were sourced as follows: Fucoidan from *Fucus vesiculosus* (F5631) and Laminarin from *Laminaria digitata* (L9634) were purchased from Sigma Aldrich (St. Louis, USA). Alginate from *Fucus vesiculosus* (ALG101), Alginate from *Laminaria japonica* (ALG100), Fucoidan from *Ascophyllum nodosum* (FUC400), Ulvan from *Ulva* spp. (ULV100), and Xylan from *Palmaria palmata* (XYL100) were purchased from Elicityl (Crolles, France).

The following was solely carried out by me: simulated human digestion of seaweed polysaccharides, 16S rRNA amplicon sequencing library preparation, qPCR, collection of data, statistical analysis, interpretation of results and write-up. P Allsopp, C Strain, and I designed the experiment. The in vitro fermentations and bacterial gDNA extractions were carried out by C Strain and I. 16S rRNA amplicon sequencing was carried out by F Crispie and the bioinformatics analysis by F Fouhy. SCFA analysis via GC-MS was carried out by me, under the supervision of T Smyth. All work relating to the faecal fermentation experiments was carried out at Teagasc Food Research Centre, Moorepark. The critical review of this chapter was conducted by me and feedback was provided by P Allsopp and E McSorley.

Chapter 7: General discussion

The analysis of results and write-up, and critical review was carried out by me. Feedback was provided by P Allsopp and E McSorley.

Chapter 1

General Introduction

1 General Introduction

1.1 Introduction

The shores surrounding the island of Ireland are habitat to multiple species of brown, green, and red edible seaweeds, which have been historically consumed by coastal communities (Monagail and Morrison, 2020). Seaweeds are also predominant in East Asian cuisine, where consumption of 5.3 g/day in Japan has been inversely correlated with chronic disease incidence (Matsumura, 2001). Whilst the nutritional properties of seaweed are well established as known sources of macronutrients (e.g. dietary fibre, protein, and lipids) and micronutrients (e.g. vitamins and minerals), there is also a suggestion that some components present in seaweed may modulate host physiology to benefit health, conducive to a functional food (Brown et al., 2014). In particular, it has been suggested that fermentation of seaweed polysaccharides by the human gut microbiota can modulate bacterial composition and metabolic output to improve host health, which is conducive to a prebiotic (Gibson et al., 2017; O'Sullivan et al., 2010). This has generated significant interest in the use of seaweeds as a source of functional fibre food ingredients with the capacity to convey health benefits through the modulation of the gut microbiota (de Jesus Raposo et al., 2016; Peñalver et al., 2020). However, owing to the high iodine contents of seaweeds, there is a concern that seaweed consumption can exceed daily upper tolerable limits for iodine (Aquaron et al., 2002; Bouga and Combet, 2015). Thus, there is in increasing interest to extract individual fibre components for functional food ingredients, including complex polysaccharide such as alginate, fucoidan, and laminarin from brown seaweeds; ulvan from green seaweeds; and agar, carrageenan, porphyran,

and xylan from red seaweeds (Charoensiddhi et al., 2016; O'Sullivan et al., 2010; Postma et al., 2017; Yuan and Macquarrie, 2015).

1.2 The human gut microbiota

The human gastrointestinal tract is colonised by an ecosystem of microorganisms that has co-evolved with humans, including approximately 10^{14} bacteria (Thursby and Juge, 2017). Approximately 160 prevalent bacterial species are reported, and dominant phyla include Firmicutes, Bacteriodetes, Actinobacteria, Proteobacteria, and Fusobacteria (Thursby and Juge, 2017). The composition of an individual's gut microbiota is usually stable beyond the first 2-3 years of life, however, inter-individual variation in gut microbiota composition, diversity, and metabolic activity exists, which is dependent on host genetics, and environmental and lifestyle factors such as diet and medication use (Conlon and Bird, 2015; Valdes et al., 2018). Differences in gut microbiota composition, diversity and metabolic output have also been implicated in human health outcomes, for instance, observational evidence shows that individuals with obesity, type 2 diabetes, and inflammatory bowel disease have lower gut bacterial diversity than healthy individuals (Valdes et al., 2018).

Methods to understand the bacterial composition and metabolic output of the human gut microbiota include the use of microbial culture, while culture-independent techniques such as 16S rRNA amplicon sequencing and whole genome shotgun metagenomics have been used to characterise microbial abundance, diversity and potential metabolic function based on microbial genomic data (Jovel et al., 2016). A combination of microbial culture and next generation sequencing has also resulted in the advent of culturomics to generate specific

conditions to identify unknown bacteria of the human gut microbiota (Lagier et al., 2018). Approaches to understand the metabolic output of the gut microbiota include the use of targeted and untargeted metabolomics (Daliri et al., 2017). This includes the identification and quantification of short chain fatty acids (SCFA), the end products of carbohydrate fermentation by the gut microbiota, using analytical techniques such as gas chromatography and mass spectrometry.

1.3 Dietary fibre and health

Dietary fibres are carbohydrate polymers that are resistant to endogenous digestive enzymes and are widely acknowledged for digestive health benefits including reduced gastric emptying, increased satiety, and increased stool mass (Anderson et al., 2009; Capuano, 2017). Dietary fibre intake (25 – 29 g/day) is also associated with a reduced risk of developing non-communicable diseases such as cardiovascular disease, diabetes and obesity (Reynolds et al., 2019). The mechanisms of which relate to the regulation of glycaemic control and cholesterol metabolism (Capuano, 2017), and increasing evidence suggests that this is mediated, in part, by the gut microbiota, owing to the fermentation of dietary fibre components in the lower gastrointestinal tract (O'Grady et al., 2019; Valdes et al., 2018). This has led to significant efforts to understand how dietary fibre influences gut bacterial composition, diversity, and metabolic output, to impact host physiology (Flint et al., 2012; Rowland et al., 2018). For instance, long-term reduction of dietary fibre intake has been associated with a loss of gut microbial diversity (Sonnenburg et al., 2016), which is associated with the onset of non-communicable disease and negative health outcomes (Valdes et al., 2018).

Undigested carbohydrates provide a substrate for saccharolytic fermentation by colonic bacteria to produce acetate, propionate and butyrate short chain fatty acid (SCFA) metabolites, with an approximate abundance of 60%, 25% and 15%, respectively (Tazoe et al., 2008). Collectively (but not exclusively), luminal SCFA reduce colonic pH which increases hostility for pathogens. SCFA are absorbed by colonocytes via passive diffusion or active transport, where they are utilised by the cell or reach the portal blood via basolateral membrane transport proteins (den Besten et al., 2013; Velazquez et al., 1997). Acetate and propionate are then utilised for hepatic cholesterol synthesis and gluconeogenesis, respectively, whilst acetate is also associated with hormonal control of satiety (promotion of CKK, PYY and GLP-1 release) (Canani et al., 2011; Frost et al., 2014; Halford and Harrold, 2012).

Butyrate is the primary colonocyte energy source widely considered a regulator of intestinal homeostasis through multiple mechanisms. For example, butyrate facilitates increased colonic blood flow and cell motility, promotes electrolyte absorption, cell differentiation/turnover and influences mammalian cell gene expression through histonedeacetylase (HDAC) inhibition, which is associated with carcinogenesis prevention (Velazquez et al., 1997). HDAC inhibition cell signalling is one of two mechanisms identified for SCFAs – the other is GPR41/GPR43 G-protein coupled receptor activation, associated with metabolism and inflammation regulation (Tan et al., 2014).

Furthermore, butyrate has a role in restoring gut barrier function via increased mucin synthesis, decreased gut permeability and immunoregulation (Leonel and Alvarez-Leite, 2012). The latter has highlighted an anti-inflammatory capacity via

Foxp3$^+$ Treg cell proliferation and NF-κB transcription factor inhibition; such that pro-inflammatory signalling mechanisms may be impaired and pro-inflammatory cytokine production can become dampened (Arpaia et al., 2013). Owing to the associated health benefits of SCFA (particularly butyrate), there is significant interest in the provision of dietary fibre to enhance colonic SCFA levels, including the use of prebiotics (Gibson et al., 2017).

1.4 Prebiotics

The concept of prebiotics was first proposed by Gibson and Roberfroid (Gibson and Roberfroid, 1995), and is currently defined as "a substrate that is selectively utilised by host microorganisms conferring a health benefit". It is suggested that many of the purported health benefits of prebiotics are mediated by the local and systemic effects of SCFA fermentation metabolites. Some reported effects of prebiotics include the inhibition of pathogens, activation of the immune system, and the microbial synthesis of vitamins (Gibson et al., 2017). Other reported health benefits of prebiotics include the reduction of diarrhoea and gut inflammation, which may hold importance in conditions such as irritable bowel syndrome, inflammatory bowel disease and colon cancer. Prebiotics are also reported to improve satiety, which may have implications in the control of obesity and related diseases such as metabolic syndrome, type 2 diabetes and cardiovascular disease (Pandey et al., 2015).

At present, the prebiotics inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are used as functional ingredients in food, beverages, dietary supplements and animal feed (Kellow et al., 2014). Only FOS and inulin have been recognised by the European Food Standards Agency (EFSA), for reducing

post-prandial glucose responses and maintaining normal defecation, respectively (Hutkins et al., 2015). Therefore, there is scope to use high-throughput *in vitro* methods to identify additional sources of potential prebiotics. This includes the use of seaweeds, given that seaweed polysaccharides have distinct chemical structures to terrestrial-derived polysaccharides.

Firstly, *in vitro* simulated digestions can be used to determine whether a putative prebiotic is resistant to gastric acid, endogenous digestive enzymes, and gastrointestinal absorption (Brodkorb et al., 2019; Gibson et al., 2004; Minekus et al., 2014; Uriot et al., 2016)**.** Secondly, static and dynamic *in vitro* gut fermentation models can be used to determine whether a candidate prebiotic is fermented by the intestinal microbiota, and whether the fermentation promotes the growth of health-associated bacteria (Gibson et al., 2004; Gibson and Fuller, 2000; Maccaferri et al., 2012; Marteau et al., 1997; Sivieri et al., 2014; Uriot et al., 2016; Wichienchot et al., 2006; Williams et al., 2015). This can be achieved by obtaining samples from the *in vitro* gut model at multiple time points to facilitate culture-dependent analysis (e.g. plating on selective media for *Bifidobacterium* spp. and *Lactobacillus* spp.)., culture-independent analysis (e.g. qPCR with *Bifidobacterium* spp. specific primers or next generation sequencing to obtain a profile of microbial diversity and relative abundance), and metabolomics analysis (e.g. GC-MS to quantify SCFA concentration).

The investigation herein forms an evaluation of the prebiotic activity of seaweeds and seaweed polysaccharides, using *in vitro* batch culture fermentation as a model of the human distal colon. Test substrates included seaweed powders and polysaccharide-rich extracts of the edible Irish seaweeds, *Fucus vesiculosus,*

Laminaria digitata, and *Palmaria palmata*; and purified alginate, laminarin fucoidan, ulvan, and xylan seaweed polysaccharides.

1.5 Aims and objectives

- Conduct a literature review on the nutritional composition, health benefits and potential risks associated with consumption of edible seaweeds.
- Conduct a literature review to evaluate the current evidence that implicates bioactive components from seaweeds with prebiotic effects.
- Evaluate the prebiotic potential of Irish brown seaweeds *Fucus vesiculosus* and *Laminaria digitata* and their extracted polysaccharide components using *in vitro* batch culture fermentation models of the distal colon.
- Evaluate the prebiotic potential of Irish red seaweed *Palmaria palmata* and its extracted polysaccharide components using *in vitro* batch culture fermentation models of the distal colon.
- Evaluate the prebiotic potential of purified seaweed polysaccharides using *in vitro* batch culture fermentation models of the distal colon. This includes the brown seaweed polysaccharides, fucoidan, alginate and laminarin; the green seaweed polysaccharide, ulvan; and the red seaweed polysaccharide, xylan.

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Chapter 2

Literature Review

Risks and Benefits of Consuming Edible Seaweeds

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2 Literature Review - Risks and Benefits of Consuming Edible Seaweeds

2.1 Abstract

Recent interest in seaweeds as a source macronutrients, micronutrients, and bioactive components has highlighted prospective application within functional food and nutraceutical industries, with impetus towards the alleviation of risk factors associated with non-communicable diseases such as obesity, type two diabetes, and cardiovascular disease.

This narrative review summarises the nutritional composition of edible seaweeds and evaluates the evidence regarding the health benefits of consuming whole seaweeds, extracted bioactives, and seaweed-based food products in humans, in balance with the determination of potential adverse effects such as excessive iodine and arsenic ingestion. If potential functional food and nutraceutical applications of seaweeds are to be realised, more evidence from human intervention studies is needed to evaluate the nutritional benefit of seaweeds and the efficacy of purported bioactive components, with mechanistic evidence imperative to substantiate health claims.

2.2 Introduction

Edible seaweeds (macroalgae) have the potential to provide a rich and sustainable source of macronutrients and micronutrients to the human diet, particularly in regions where seaweed makes a significant contribution to regular meals, for example, in Japan, where approximately one fifth of meals contain seaweed (Lange et al., 2015; MacArtain et al., 2007; Rebours et al., 2014). Inclusion of seaweeds in western diets has traditionally been limited to artisanal practises and coastal

communities, but has gained wider consumer interest in recent years, courtesy of the health-food industry (Bouga and Combet, 2015). The recent surge of interest in seaweed is fuelled by attention towards the bioactive components from seaweed, which have potential applications in the lucrative functional food and nutraceutical industries, with impetus towards the alleviation of metabolic risk factors such as hyperglycaemia, hypercholesterolaemia, and hyperlipidemia, as well as anti-cancer activity and anti-microbial function (Collins et al., 2016). The candidate bioactive components of interest to industry include isolated polysaccharides (e.g. alginate, fucoidan), proteins (e.g. phycobillirubins), polyphenols (e.g. phlorotannins), carotenoids (e.g. fucoxanthin), and omega-3 long chain polyunsaturated fatty acids (e.g. eicosapentaenoic acid). The focus of scientific experiments and human studies to date has predominantly focused on brown seaweeds and derivatives, owing to their commercial abundance and perceived sustainability.

Despite the nutritional attributes of red seaweeds such as *Porphyra spp.* (nori) or *Palmaria palmata* (dulse) with high protein content, there are relatively few investigations which have focused upon red seaweeds as a source of bioactives, albeit an abundance of *in vitro* studies and *in vivo* animal studies represent progress towards understanding their heath promoting activities. There are limited reports of green seaweeds contributing to the human diet or as a source of bioactives, despite the potential to exploit transient algal blooms (Postma et al., 2017).

Aquaculture is recognised as the most sustainable means of seaweed production and accounts for approximately 27.3 million tonnes (96%) of global seaweed production per annum, yet, the growing demand for seaweed based food

ingredients has called for more established guidelines and regulations to ensure sustainability (Mac Monagail et al., 2017). Future considerations for stakeholder management include best practises for cultivation, resource ownership, harvesting rights/licencing, certification/validation, over-exploitation, biomass regrowth, environmental impacts, and the development of a sustainable value chain within the agri-food sector (Rebours et al., 2014; Tiwari, 2015).

An abundance of seaweed products currently available to purchase, including both whole seaweed and seaweed extracts, are marketed both directly and indirectly as value-added products for the promotion of health in the supplement market. The health claims associated with seaweed products are often based on insufficient (or completely absent) evidence from human intervention studies to substantiate such statements. Furthermore, there are considerable safety concerns in relation to potential adverse events associated with seaweed consumption, particularly in light of the variable and potentially dangerously high concentrations of iodine and heavy metals (including arsenic species) in certain seaweeds (Holdt and Kraan, 2011; Suleria et al., 2015). There is currently limited legislation to enforce food or supplement companies to disclose mineral, heavy metal or iodine content of seaweed products or provide guidance on a safe portion size of certain whole seaweeds in order to prevent excessive intakes (Bouga and Combet, 2015). Ultimately, if the nutrients present in seaweeds shall contribute towards future global food security, either as a feed ingredient or directly as a food, the industry should develop a sustainable heavy metal/iodine monitoring programme or alternatively identify novel processing technologies to ensure that unsafe components such as arsenic are minimised to achieve safe levels and thus protect

the food chain (European Commission, 2009; FAO, 2016; Kraan, 2013; Rebours et al., 2014; Republic of the Philippines Ministry of Natural Resources, 1983).

The health benefits of seaweed beyond the provision of essential nutrients has been supported by *in vitro* studies and some animal studies; however, many of these studies have inappropriate biomarkers to substantiate a claim and have not progressed to suitably designed human intervention trials to evaluate efficacy. The limited evidence that does exist makes some seaweed components attractive as functional food ingredients, however, more human evidence is needed to evaluate the nutritional benefit conferred and the efficacy of purported bioactives (with mechanistic evidence) in balance with determination of potential adverse effects. Through an evaluation of the nutritional composition of edible seaweeds, this review summarises the available evidence and outlines potential risks alongside the health benefits of consuming whole seaweeds, extracted bioactives, and seaweed-based food products in humans, and identifies future opportunities for functional food and nutraceutical applications.

2.3 Nutritional composition

A number of edible seaweeds are recognised as novel foods in Europe, albeit the nutritional composition of brown, red and green seaweeds vary between species, season, and ecology of the harvesting location (European Commission, 2018). Therefore, there is a need to characterise the composition of seaweeds in relation to the influence of location and seasonality on seaweed content. There are continuing efforts to catalogue such information on the variability of nutritional composition which will facilitate the identification of optimal harvesting periods and/or locations for a given species which would be of interest to the functional

food industry for targeting optimal conditions for isolating target bioactives (Madden et al., 2012; Schiener et al., 2015; Soares et al., 2017). **Table 2-1**, **Table 2-2** , and **Table 2-3** present the macronutrient content of multiple brown, red, and green seaweeds, respectively, and consider a 5g serving relative to reference nutrient intakes. **Table 2-4** and **Table 2-5** present the same nutritional information for a selection of dried seaweed products available to purchase throughout the UK and the Republic of Ireland, to provide context regarding the current market, and to compare with the compositional studies discussed.

2.3.1 Protein

The protein content of seaweed has gained considerable attention given the emerging challenges to food security in relation to identifying alternative and sustainable protein sources (Harnedy and FitzGerald, 2011). As outlined in **Table 2-1**, **Table 2-2**, and **Table 2-3**, the protein content ranges from 5.02% to 19.66% in brown seaweeds; from 0.67% to 45.00% in red seaweeds; and from 3.42% to 29.80% in green seaweeds; where a 5g portion of dried seaweed corresponds to a maximum of 1.97%, 4.50%, and 2.98% of the recommended nutrient intake for protein, for brown, red, and green seaweeds, respectively. On a gram for gram basis, seaweeds have comparable protein and amino acid contents to beef, however, seaweeds are consumed in much smaller quantities (Greenwood et al., 1951). It should also be noted that seaweed protein content is often derived from total nitrogen using a conversion factor of 6.25 (Kjeldahl method), which is likely an overestimate given the non-protein sources of nitrogen in seaweed. Hence, species specific conversion factors for seaweed, ranging from 3.57 – 5.72 have been proposed (Lourenço et al., 2002). The amino acid composition of proteins is critical

to determining its value to the human diet, particularly in achieving an adequate intake of essential amino acids. However, the digestibility of seaweed protein within the gastrointestinal tract will significantly impact its nutritional value, with protein-polysaccharide interactions significantly reducing digestion efficiency.

An overview of the amino acid contents of several brown, red, and green seaweeds is presented in **Table 2-6**, **Table 2-7**, and **Table 2-8**. Seaweeds offer a source of lysine, an essential amino acid often limited in terrestrial plant protein sources such as corn, maize, soy, rice, and wheat (EFSA, 2012; Misurcova et al., 2014; Qasim, 1991). It is reported that an 8g portion of *Palmaria palmata* contains up to 21.9% cysteine recommended daily intake, however, seasonal variation in the total protein content of *Palmaria palmata* is evident, reported as 21.9% in winter-spring compared to 11.9% in summer-autumn, with 26 – 50% comprising of essential amino acids (Galland-Irmouli et al., 1999; Misurcova et al., 2014). Thus, exploiting seaweeds as non-animal protein sources may be possible through harvesting plans, which optimise protein and amino acid contents.

The digestibility of seaweed protein has been estimated using *in-vitro* methods, and is reported as: *Fucus vesiculosus* = 14.7%, *Laminaria digitata* = 16.9%, *Undaria pinnatifida* = 28.0%, *Chondrus crispus* = 45.0%, *Porphyra tenera* = 69.4%, *Palmaria palmata* = 56%, *Porphyra columbina* = 74.3% (Cian et al., 2014; Galland-Irmouli et al., 1999; Goñi et al., 2002). The digestibility of protein from *Undaria pinnatifida and Porphyra tenera* in rodents is reported as 86.1% and *86.2%,* respectively; while the digestibility of *Undaria pinnatifida* protein in *humans is reported as* 70.0%, which is similar to the protein digestibility of land based plants (Cerna, 2011; Urbano and Goni, 2002; Yamada et al., 1991). Although *in vivo* digestibility data

suggest that seaweed protein is bioaccessible, protein-polysaccharide interactions within the seaweed matrix could prevent enzyme-substrate complex formation and hinder proteolysis of seaweed proteins. Indeed, xylanase and cellulase polysaccharide enzymatic treatments have improved *Palmaria palmata* protein bioaccessibility 1.7-fold and 3-fold, respectively, which may favour the use of protein extracts to maximise protein and amino acid intake from seaweeds, with food, feed, supplement, and nutraceutical applications (Beasley et al., 2013; Cerna, 2011; Maehre et al., 2016). Methods for protein extraction from brown, red and green seaweeds are described comprehensively elsewhere, where the use of proteolytic and saccharolytic enzymes such as Celluclast® or Shearzyme® (Novozymes, Copenhagen, Denmark) are reported to improve protein extraction yield and endogenous digestion (Bleakley and Hayes, 2017; Harnedy and FitzGerald, 2015, 2013, 2013; Kadam et al., 2017). Hitherto, the bioactivities pertained to peptides extracted from red seaweeds such as *Palmaria palmata* and *Porphyra spp.,* and brown seaweeds such as *Undaria pinnatifida* include antihypertensive, antioxidant, and antidiabetic effects (Admassu et al., 2018). This includes cardio-protective effects such as reduced blood pressure via angiotensin converting enzyme inhibition, anti-diabetic activity via dipeptidyl peptidase IV inhibition, and promotion of iron absorption; as well as potential application as food preservatives owing to anti-oxidant capacities (Cian et al., 2016; Fitzgerald et al., 2014; Harnedy et al., 2017, 2015; Jimenez-Escrig et al., 2011; Sato et al., 2002; Suetsuna et al., 2004). Therefore, seaweed protein extracts hold promise as a seaweed-based protein source, providing bioactivity is validated in humans.

It is estimated that 56 million metric tonnes of algae will be required per annum as

an alternative protein source by 2054, which will represent 5.94% of global protein demand (Probst, 2015). Given the variability of protein content and bioavailability of protein from whole seaweeds, protein extracts may offer a substantial contribution to non-animal protein sources in the future.

2.3.2 Dietary fibre

Many populations are failing to meet daily requirements for dietary fibre (EFSA, 2010a; FDA, 2017; Scientific Advisory Committee on Nutrition, 2015). The potential functional properties of dietary fibre are associated with its viscous and water binding properties within the gastrointestinal tract, which has been suggested to promote satiety and weight loss; delay gastric emptying to improve glycaemic control; enhance stool bulking to reduce gut transit time and increased defecation frequency; and enhance bile acid excretion resulting in reduced blood LDL cholesterol (Clark and Slavin, 2013; EFSA, 2011a). Dietary fibre components are also suggested to improve health through their fermentation by the colonic microbiota, which can favourably alter gut microbial composition and enhance the production of health-associated volatile fatty acids such as acetate, propionate, and butyrate. The fibre induced alterations to microbiota composition and the associated metabolites produced are increasingly associated with the promotion of gastrointestinal, cardiometabolic, immune, bone, and mental health (Gibson et al., 2017).

Owing to the range of pertained beneficial health effects associated with dietary fibre consumption, there is increasing interest from the food industry to identify sustainable, alternative sources of dietary fibre (Clark and Slavin, 2013; EFSA, 2011a). The high fibre content of seaweed makes it a promising candidate.

However, the contribution of consuming whole seaweed to the current recommended dietary fibre intake of 25g/day is limited, with a 5g serving of brown, red, and green seaweeds contributing up to 14.28%, 10.64%, and 12.10% of dietary fibre intake, respectively (**Table 2-1**, **Table 2-2**, and **Table 2-3**) (EFSA, 2010a). This has led to increasing interest towards industrially applicable extraction and isolation of individual fibre components from seaweed. The range of fibre components in seaweed is diverse with brown seaweeds known to contain alginate, laminarin, and fucoidan polysaccharides; red seaweeds contain agar, carrageenan, porphyran, and xylan; whilst green seaweeds contain ulvan, xylan, and cellulose (Brownlee et al., 2005; Devillé et al., 2007; Lahaye and Robic, 2007; Lahaye and Rochas, 1991; Li et al., 2008; Usov, 2011).

Whilst industry has used some seaweed derived fibres (alginate, carrageenan, and agar) for decades for their emulsifying, stabilising and thickening characteristics to improve the sensory properties of food, there is limited interest on their impact as functional dietary fibre ingredients. The existing widespread use of these seaweed derived fibre components in the food industry has ensured that they are deemed to be safe for human consumption according to EFSA and the FDA, thus the use of alginate isolated from brown seaweeds by the food industry makes it a leading candidate for application in the functional food market (EFSA, 2017a, 2016; FDA, 2016a). The recent novel food classification of fucoidan by EFSA also makes it an emerging functional food ingredient candidate, whilst suggestions that low molecular weight carrageenan components (<50 kDa) may negatively impact health (pro-inflammatory), have tempered interest in its potential as a functional ingredient (Burges Watson, 2009; European Commission, 2017). Other seaweed

fibres such as xylan, laminarin, and ulvan, have not received official EFSA approval, thus more research is needed to ascertain whether these carbohydrates are safe for human consumption, and the designated safety of these seaweed derived fibre components will make them ready for the food market as a nutritional ingredient, providing associated health claims are substantiated.

The majority of research in humans regarding the health benefits of seaweed derived dietary fibre components have focused on the potential anti-obesogenic effects, including improved satiation, delayed nutrient absorption, and delayed gastric emptying, but the effects of whole seaweeds containing alginate appear to be limited (Brownlee et al., 2005; Chater et al., 2015; El Khoury et al., 2015). Several human intervention placebo-controlled trials have shown alginate consumption to significantly impact appetite and food intake. An acute study by Peters *et al.* (Peters et al., 2011) showed that consumption of an alginate drink enhanced self-reported satiety and reduce feeling of hunger in a dose dependent manner compared to the placebo control. Another parallel study in overweight men showed consumption of a *Ascophyllum nodosum* enriched (4%) bread reduced energy intake by 109 and 506 kCal at 4 and 24-hours post consumption compared to an isocaloric placebo and similar results were reported when consumption of a preload alginate drink reduced energy intake of 44kcal following an ad libitum lunch (Georg Jensen et al., 2012; Hall et al., 2012). A crossover study has reported a reduced daily energy intake of 135 kcal when consuming alginate (1.5g/100ml) prior to meals, but no significant effect of a preload alginate drink on measures of energy intake, or concentration of satiety hormones in overweight/obese individuals is reported elsewhere (Odunsi et al., 2010; Paxman et al., 2008). The

role for alginate on appetite and food intake would appear to be convincing, albeit research is needed to the characterise the efficacy of alginates taking into consideration the relationship between structure and function (particularly molecular weight and the guluronate:mannuronate ratio) and the role of the gelling capacity of the alginate. Furthermore, research into the mechanism of action is required given the lack of effect of alginate on gastric emptying (Georg Jensen et al., 2012; Odunsi et al., 2010). The formulation of alginate food products that are organoleptically acceptable to the consumer is another consideration to industry. Longer term studies are required to provide information of these benefits on appetite control and weight management.

There is also considerable interest in the role of alginate on glycaemic control, particularly its impact on postprandial glucose absorption. A review of the evidence by EFSA concluded that sodium alginate failed to reduce post-prandial glycaemic responses without a disproportionate increase in post-prandial insulinaemic responses and thus a health claim was rejected (Torsdottir et al., 1991; Wolf et al., 2002). Other fibres such as beta glucan have received favourable EFSA opinions for its ability to reduce postprandial glucose absorption which has been attributed to a reduction in the rate of gastric emptying (EFSA, 2011b). The effect of alginate on glucose metabolism needs to be further investigated; particularly the impact it may have on postprandial insulinaemic response.

The recent designation of fucoidan with FDA GRAS status and EU novel food status along with the accumulating *in vitro* and *in vivo* evidence of its potential anti-obesogenic effects make it an attractive ingredient for the functional food industry (European Commission, 2017; FDA, 2016b; Kim et al., 2014; Lim et al., 2017).

Nevertheless, only one human study has investigated the anti-obesogenic effects of seaweed derived fucoidan. A randomised double blind parallel placebo controlled trial in an overweight/obese cohort showed that participants who consumed fucoidan (500mg/day) for three months had significantly reduced diastolic blood pressure and LDL cholesterol compared to the placebo control (Hernandez-Corona et al., 2014). No changes in weight, waist circumference, BMI, adiposity, systolic blood pressure, total cholesterol, HDL cholesterol, blood glucose, or blood triglyceride were observed, however, blood insulin and insulin resistance (HOMA-IR) were increased following the fucoidan intervention compared to baseline, but not compared with the placebo group. The authors suggested that fucoidan consumption downregulated PPARγ transcription factor expression to suppress adipocyte differentiation and insulin signalling.

Conversely, evidence from animal models of obesity and diabetes suggest that low molecular weight fucoidan could ameliorate dyslipidemia and improve insulin sensitivity through the activation of insulin signalling pathways in adipocytes and hepatocytes (Jeong et al., 2013; Kim et al., 2014; Lin et al., 2017; J. Wang et al., 2013; Yokota et al., 2016).

Further evidence is required from human intervention trials to understand how dietary fucoidan may modulate host glucose and lipid metabolism to exert anti-obesigenic and anti-diabetic effects. This may also require an understanding of how the molecular weight of fucoidan affects bioactivity.

Fucoidan is also reported to have anti-coagulant properties, serving as a catalyst for anti-thrombin mediated and heparin cofactor II–mediated thrombin inhibition (Church et al., 1989; Dockal et al., 2011; Irhimeh et al., 2009). Oral administration

of *Undaria pinnatifida* extract (9g/day) with 75% fucoidan (MW ~713KDa) for twelve days increased activated partial thromboplastin time, decreased thrombin time, and increased antithrombin-III compared with placebo, albeit the authors concluded the improvements were small and that the impact of oral delivery may be limited (Irhimeh et al., 2009). Another study investigated oral administration of 400mg/day fucoidan (extracted from *Laminaria japonica*, MW ~300kDa) for five weeks and reported a significant reduction in thrombus lysis time, albeit fucoidan was not detected in subjects' blood using a fucoidan specific monoclonal antibody ELISA (Ren et al., 2013). This suggests that fucoidan may not be bioavailable in humans, although fucoidan has been demonstrated to be absorbed through the small intestine using *in vitro* and *in vivo* experiments (Nagamine et al., 2014). The anticoagulant properties of fucoidan are gaining attention by the pharmaceutical industry; however its anticoagulant function as a food ingredient and its potential impact on health will require much more human evidence to verify both safe and efficacious doses particularly with consideration to individuals on anticoagulant therapy.

There is also a wealth of evidence to support an anti-cancer function of fucoidan, however, the majority of evidence is from *in vitro* studies and animal studies and is reviewed elsewhere (Atashrazm et al., 2015). A recent clinical trial in colon cancer patients investigated the impact of consuming a fucoidan (4g twice daily) supplement alongside their chemotherapy (Tsai et al., 2017). The study showed that the patients consuming fucoidan had a significantly better disease control rate compared to placebo control, however, did not show any change in overall response rate, progression-free survival, overall survival, adverse effects, and

quality of life. Whilst the effect of fucoidan on disease control rate is a notable benefit, its role in contributing to cancer treatment remains unknown. While the advertisement of cancer treatments is prohibited in the UK, the legislation regarding cancer prevention is less (Cancer Act 1939, 1939; The Consumer Protection from Unfair Trading Regulations 2008, 2008). Further, marketing fucoidan as a food ingredient with cancer prevention effects is extremely difficult to substantiate and may prove a very difficult regulatory environment to navigate.

There is increasing interest in the potential prebiotic effect of seaweed derived fibre through its effect on the composition and metabolism of the colonic microbiota and how fibre fermentation may impact human health. A range of *in vitro* faecal batch culture studies have demonstrated the fermentability of seaweed fibre components with noted increased production of short chain fatty acids and modulation of gut microbial communities (Bai et al., 2017; Bajury et al., 2017; Fu et al., 2018; Michel et al., 1996; Ramnani et al., 2012). Whilst animal studies have confirmed gut microbiota modulation and short chain fatty acid production, the health benefits are only attributed to weight management thus far (Devillé et al., 2007; Liu et al., 2015; Nguyen et al., 2016). There is a lack of human intervention trials which have investigated the fermentability of seaweed fibre components, and their potential to impact health outcomes associated with prebiotics (Gibson et al., 2017).

2.3.3 Fat

The fat content of seaweed tends to be low relative to total dry weight, where the % fat content is highest in winter, lowest in summer, and fatty acid composition varies dependent on season (Gressler et al., 2011; Madden et al., 2012; Yamada et

al., 1991). An example of the latter includes *Saccharina latissima* grown in integrated multi-trophic aquaculture, where lipid concentration and polyunsaturated fatty acid (PUFA) content was highest in March and November, yet lowest in January (Marinho et al., 2015). In terms of nutritional contribution, it is reported that seaweed-derived lipids have good digestibility, for example, up to 98% of *Undaria pinnatifida* fat contents (1.5% DW) are digestible in adults (Yamada et al., 1991).

Table 2-1, **Table 2-2**, and **Table 2-3** present the total fat content of several brown, red, and green seaweeds, respectively, while **Table 2-9**, **Table 2-10**, and **Table 2-11** present a breakdown of lipid contents. Total lipid contents range from 0.29% in *Sargassum polycystum* to 8.88% in *Porphyra spp.* (Matanjun et al., 2009; Paiva et al., 2014). *Porphyra spp.* have the lowest saturated fatty acid (SFA) content (17.4% of total fatty acids, FA), whereas SFAs constituted 74% of total FA in *Plocamium Brasiliense* (Gressler et al., 2011). Monounsaturated Fatty Acid (MUFA) content relative to total FA content ranged from 3.3% in *Ochtodes secundiramea* to 47.1% in *Fucus vesiculosus* (Maehre et al., 2014). PUFA content of total FA ranges from 6.7% in *Ulva lactuca* to 69.1% in *Undaria pinnatifida* (Sanchez-Machado et al., 2004; Yaich et al., 2011). This sample also had the highest PUFA/SFA ratio (3.39), while the three *Palmaria* species recorded demonstrate the lowest n-6/n-3 ratio, whereas *Gracilaria gracilis* had the highest (Francavilla et al., 2013).

Dietary reference values are not set for PUFAs collectively, but an adequate intake of 4% energy intake is recommended for n-6 linoleic acid (EFSA, 2010b). Foods with a greater ratio of PUFAs, relative to SFAs, may be favourable, in order to maintain blood LDL-cholesterol within normal concentrations, although more human

intervention studies are needed to confirm efficacies of PUFAs upon dyslipidemia management and the attenuation of low-grade inflammation (EFSA, 2011a; Hunter and Hegele, 2017).

Evidence for bioactive efficacies specific to seaweed lipids is limited, although male KK-Ay mice treated with 1% *Undaria pinnatifida* lipid were seen to have a significant reduction in body weight after four weeks when compared to the control group, whilst total white adipose tissue weight was reduced in mice who consumed both the *Undaria_pinnatifida* lipid and n-3 polyunsaturated fatty acid rich scallop phospholipids (Okada et al., 2011). Other anti-inflammatory activities of seaweed lipids concern the inhibition of LPS-induced inflammation in human THP-1 macrophages by lipids derived from the red seaweeds *Porphyra dioica, Palmaria palmata* and *Chondrus crispus*, while lipids extracted from *Gracilaria spp.* inhibited LPS-induced nitric oxide production in murine RAW 264.7 macrophage cells and decreased cell viability in human T-47D breast cancer cells and 5637 human bladder cancer cells (da Costa et al., 2017; Robertson et al., 2015). An anti-cancer effect of a C18 fatty acid extracted from *Ulva lactuca* was also reported, via the activation of the Nrf2-ARE pathway to promote ROS scavenging (R. Wang et al., 2013).

Given that whole seaweed consumption is unlikely to make a significant contribution to dietary fat intake, owing to low lipid contents, macroalgae may offer a sustainable source of extractable PUFAs for further investigation regarding the anti-inflammatory effects to ameliorate obesity and associated co-morbidities. This may have prospective supplement or nutraceutical applications.

2.3.5 Polyphenols

Polyphenols are highly complex cell wall structural components, often bound to cell wall polysaccharides, and function to protect against oxidative damage (Heffernan et al., 2015). A diverse number of flavonoid and phlorotannin polyphenols are abundant in brown seaweeds, which vary in structure, molecular weight, and level of isomerisation (Hwang and Thi, 2014; Murugan et al., 2015).

The purported bioactivities of seaweed polyphenols include potential anticancer activities, antioxidant activities, and digestive enzyme inhibition, where the latter may prevent lipid absorption and maintain glucose homeostasis (Farasat et al., 2014; Machu et al., 2015; Murugan et al., 2015; Shanura et al., 2016; Wan-Loy and Siew-Moi, 2016; H. C. Yang et al., 2010).

The bioavailability of polyphenolic compounds in food varies greatly, but it is understood to be low (Bohn, 2014). There is currently a lack of information regarding the bioavailability of seaweed derived polyphenolic compounds, however, a recent human intervention trial investigated the bioavailability of polyphenols extracted from *Ascophyllum nodosum*, provided initial indications of interpersonal variation in polyphenol uptake, with 0.011 – 7.757 µg/ml of polyphenols detected in the serum, whilst total urinary phlorotannin and metabolite concentration ranged from 0.15 – 33.52 µg/ml (Corona et al., 2016). The authors concluded, based on the rate of absorption (6-24 hours), that gut microbiota-mediated metabolism of the polyphenols could be a major contributor to the apparent interpersonal variation of polyphenol absorption. Consequently, more human studies are needed to investigate the bioavailability of polyphenolics when consuming whole seaweeds, as there is potential for seaweed-derived

fermentable fibres and polyphenols to exert synergistic effects on the gut microbiota and the host. There is also scope for more fundamental research to further characterise the role of the gut microbiota on phlorotannin metabolism, which could significantly impact associations with health benefits.

There is considerable evidence from animal studies to support a role for seaweed polyphenols to impact glucose and lipid digestion and metabolism, suggested to have potential in preventing diabetes and obesity associated complications. Diabetic rats fed an ethanol extract (150 and 300mg/kg) or a water extract (300mg/kg) from *Sargassum polycystum* significantly reduced blood glucose, glycated haemoglobin, total cholesterol, blood triglycerides and plasma atherogenic index in diabetic rats (Motshakeri et al., 2014, 2013). Polyphenols from both *Ecklonia stolonifera* and *Ascophyllum nodosum* have been shown to favourably alter glucose and insulin metabolism in diabetic mouse models, whilst *Ecklonia cava* polyphenols significantly reduced serum and liver triglycerides and total cholesterol in a diabetic mouse model (Iwai, 2008; Kim and Kim, 2012; Zhang et al., 2007). Another study showed that inclusion of *Gelidium amansii* phenoic-rich extract reduced blood glucose and serum insulin in diet-induced obese mice, and protected against the adverse effects of diet-induced obesity in mice via decreased blood triglycerides and total cholesterol (Kang et al., 2016). Hitherto, the mechanism of action remains elusive, but current evidence supports a role for the inhibition of digestive enzymes including α-amylase, α-glucosidase, and lipase (Austin et al., 2018; Chater et al., 2016; Pantidos et al., 2014; Zhang et al., 2007).

There is limited evidence for the efficacy of seaweed polyphenols to exert anti-obesigenic effects or to maintain glucose homeostasis in healthy humans. For

example, consumption of 500 mg seaweed extract with ≥ 10% polyphenols did not improve postprandial glucose concentration after a 50g carbohydrate load (bread) compared to the placebo, but lowered plasma insulin incremental area under the curve in healthy adults (n = 23) (Paradis et al., 2011). Elsewhere, neither a 500 mg nor 2000 mg dose of *Fucus vesiculosus* polyphenol-rich extract reduced postprandial glucose or insulin responses beyond that of the cellulose placebo after a 50g carbohydrate load (white bread) in healthy adults (n = 38) (Murray et al., 2018b).

In Korean adults with increased cholesterol, however, a significant reduction in total cholesterol, LDL cholesterol, and C-reactive protein was observed compared to baseline, following the treatment of 400mg/day *Ecklonia cava* polyphenol extract for 12 weeks (Lee et al., 2012). Potential mechanisms of action may be associated with the inhibition of adipogenesis, where dieckol is demonstrated to down-regulate AMPK signalling in 3T3-L1 preadipocytes (Ko et al., 2013).

A recent meta-analysis concluded that polyphenol-rich marine extracts could reduce fasting blood glucose, total cholesterol, and LDL cholesterol in humans, but a paucity of human intervention studies has led to inconsistent findings for the effect of seaweed polyphenols upon other biomarkers associated with type two diabetes and cardiovascular disease risk, including postprandial blood glucose, fasting insulin, HDL cholesterol and triglycerides (Murray et al., 2018a).

With *Ecklonia cava* phlorotannins deemed safe for food supplement use in the EU, further evidence in healthy and at-risk human populations is required to ascertain the bioactivities of seaweed polyphenols, while efforts to optimise polyphenol extraction procedures will be crucial to maximise their potential as food

ingredients (EFSA, 2017b; Gall et al., 2015; Li et al., 2017; Murray et al., 2018b; Yoon et al., 2017).

2.3.6 Fucoxanthin

Carotenoids are a group of tetrapenoid compounds in seaweeds that contribute to photosynthesis and their antioxidant properties facilitate protection from UV damage. The main carotenoid present in seaweeds with potential application in the food industry is fucoxanthin, extracted from brown seaweeds (Christaki et al., 2013; Mikami and Hosokawa, 2013). Evidence suggests that fucoxanthin may have potential as a food preservative to prevent lipid peroxidation in meat through its antioxidant activities (Sellimi et al., 2017).

Previous research regarding fucoxanthin has focused on its potential as a functional food ingredient to reduce diabetes and obesity risk, albeit the evidence is predominantly derived from *in vitro* and animal studies. Fucoxanthin is thought to mediate its effects through the inhibition of α-amylase and α-glucosidase digestive enzymes, and impact lipid metabolism through the modulation of leptin and adiponectin, which results in a downregulation of lipogenesis and an upregulation lipolysis (Harris, 2014; Nagappan et al., 2017). A range of *in vivo* animal studies have shown fucoxanthin supplementation to reduce: body weight gain, lipid accumulation in the liver, blood glucose and plasma insulin, decreased insulin resistance and also improve plasma lipid profile (Maeda et al., 2008; Muradian et al., 2015). The impact of fucoxanthin on cholesterol metabolism in mice differs, where Beppu *et al*. (Beppu et al., 2012) reported increased serum HDL and non-HDL cholesterol and serum total cholesterol, whilst Jeon *et al*. (Jeon et al.,

2010) reported decreased serum cholesterol and increased fecal cholesterol following fucoxanthin diets.

The anti-obesogenic effects of fucoxanthin have been reported in a human intervention trial in which consumption of fucoxanthin over four weeks significantly decreased BMI, body weight, and visceral fat area in mildly obese adults (BMI 25 – 30 kg/m^2), with no adverse events reported, however, “mixed tocopherol” and “kelp extract” components were included in each capsule (Hitoe, 2017). There is evidence to support a role for a proprietary product containing brown seaweed fucoxanthin, omega-3 fatty acids and punicic acid to exert anti-obesigenic effects (Abidov et al., 2010). Consuming the fucoxanthin product over 16 weeks significantly reduced bodyweight by 5.5kg and 5kg in premenopausal woman with non-alcoholic fatty liver disease (NAFLD) and normal liver fat (NLF), respectively, in comparison to the placebo. Furthermore, statistically significant improvements in liver fat content, systolic and diastolic blood pressure, and CRP were recorded in both cohorts consuming the fucoxanthin product, but not for the placebo; whilst significantly reduced waist circumference and serum triglycerides occurred in the Xanthigen-NAFLD group only. Interpretation of the role of fucoxanthin in this study is confounded by the additional components (omega-3 fatty acids, punicic acids derived from pomegranate seed oil) also present in the treatment, thus more effort is needed to verify such activity with fucoxanthin alone.

There is a need to undertake more research in healthy human participants to determine the role of fucoxanthin in altering lipid metabolism and its potential to reduce the risk of obesity. There is also scope to investigate other carotenoids

present in red seaweeds, such as lutein, β-carotene, and zeaxanthin, and carotenoids present in green seaweeds, such as lutein, β-carotene, echinenone, violaxanthin, neoxanthin, for their potential anti-obesigenic, anti-diabetic, or anti-oxidant bioactivities (Bjornland and Aguilarmartinez, 1976; Owen, 1954; Parjikolaei, 2016; Pirian et al., 2016; Takaichi, 2011).

As with polyphenols, cost-effective and scalable extraction protocols must be developed to produce sufficient quantities of fucoxanthin for clinical trials which assess bioactive efficacy and mechanism of action, and for prospective application as food ingredients or supplements.

2.4 Micronutrients

Several studies indicate that seaweed consumption contributes to dietary mineral intake, and a higher intake of foods containing seaweed has been associated with sufficient calcium intake to prevent osteoporosis in Korean postmenopausal women (Desideri et al., 2016; Lim et al., 2015; MacArtain et al., 2007; Ruperez, 2002). In contrast, one report indicates no meaningful contribution to dietary intake of Na, K, Mg, P, Ca, Fe, Mn, Zn, Se or Cu, when considering a portion size of 5g/day DW of 17 brown seaweed and 17 red seaweed food products sourced from China, Japan, and South Korea (Dawczynski et al., 2007). This suggests great differences in mineral content between sources, and the data presented in **Table 2-12**, **Table 2-13**, and **Table 2-14** reiterate this.

Seaweed could be important as a source of iron, where *Sargassum spp*. is reported to contain 156.9 mg iron per 100g DW, and the addition of this seaweed to both wheat and maize based bread increased the proportion of absorbed iron (Garcia-

Casal et al., 2009). Elsewhere, *Sargassum spp*, improved iron absorption of a rice meal, where the iron content ranged from 81 – 290 mg/100g dry weight over 12 months (highest iron content in July and the lowest in January) (Garcia-Casal et al., 2007).

Seaweeds are also considered a rich source of magnesium, but the bioaccessibility of magnesium varies between seaweeds, where *Ulva pertusa*, *Laminaria japonica* and *Gloiopeltis furcate* contained 10.47 mg/kg (41.8% bioaccessible), 6.55mg/kg (60.8% bioaccessible), and 8.18mg/kg (72.5% bioaccessible) magnesium, respectively, under simulated gastrointestinal conditions (Nakamura et al., 2012). A subsequent mouse study found that magnesium from *Laminaria japonica* was absorbed most efficiently, which indicates that magnesium intake from seaweed will vary between sources.

2.4.1 Vitamins

Multivitamin supplements are commonly used in the general population to achieve recommended daily intakes, whereas seaweeds may represent an abundant source of both fat and water-soluble vitamins, as outlined in **Table 2-15**, **Table 2-16**, and **Table 2-17**. For example, the vitamin A content (retinol equivalents of carotenoid content, determined by HPLC) of a 5g dried portion varies from 14.5µg (2% RNI) in *Ulva rigida* to 70.5µg in *Fucus spiralis* (10% RNI) (Paiva et al., 2014; Taboada et al., 2010). The vitamin C content varies from 0.41mg (1% RNI) in *Ascophyllum nodosum* to 9.24mg (23% RNI) in *Undaria pinnatifida* (MacArtain et al., 2007). Reported folate (vitamin B9) content varies from 7.5µg (3.75% RNI) in *Ulva spp*. to 5400µg in *Ulva rigida* (2700% RNI) (MacArtain et al., 2007; Taboada et al., 2010). Both

seasonal and geographical variation may explain such variation within the same genus.

Only one study to date has analysed the vitamin D3 content of seaweeds, which reported 0.83mg/100g DW in *Fucus spiralis* and 1.05mg/100g dry weight in *Porphyra spp.*(Paiva et al., 2014). This equates to 41.5µg (415% RNI) and 63.5µg (635% RNI) per 5g dried portion in *Fucus spiralis* and *Porphyra spp*., respectively (European Parliament, 2011). Further characterisation studies are therefore required to corroborate such findings which suggest seaweed is a valuable dietary source of vitamin D.

Seaweed is one of the few non-animal sources of vitamin B12, where *Enteromorpha spp*. and *Porphyra spp*. are reported to contain 63.58µg and 32.26µg per 100g DW, respectively (Watanabe et al., 1999). Elsewhere, *Porphyra spp.* contained 6.69µg (446% RNI), while other reports of vitamin B12 content of seaweeds do not specify whether it is present in the active form that can be absorbed and utilised in humans (MacArtain et al., 2007; Miyamoto et al., 2009). Seaweeds containing active vitamin B12 may favour individuals following a vegan diet, who are at risk of vitamin B12 deficiency, where the authors of a cohort study in children following a vegan diet for 4 - 10 years attributed healthy vitamin B12 status to Nori consumption (*Porphyra spp.*) (Pawlak et al., 2014; Suzuki, 1995). Other seaweeds recorded in this study, such as hijiki, wakame, and kombu are understood to contain limited amounts of vitamin B12, or contain vitamin B12 analogues that, because of structural differences, do not have vitamin B12 activity in humans (Watanabe et al., 2002). It is also reported that drying *Porphyra spp.*

inactivates vitamin B12, therefore processing methods may impact vitamin bioavailability (Yamada et al., 1999).

In summary, seaweeds represent a source of both fat and water-soluble vitamins, where consumption may improve vitamin status. Owing to inter-species, seasonal and geographical variation in seaweed vitamin contents, however, characterisation of vitamin contents is required for prospective seaweed supplements, and there are a limited number of human studies, with few participants, that have investigated the bioavailability and activity of vitamins obtained from seaweeds.

2.4.2 Salt

According to the National Diet and Nutrition Survey: Assessment of dietary sodium, adults aged 19 to 64 years consume, on average, 7.8 g, 8.0 g, and 8.6 g salt per day in Scotland, England, and Northern Ireland, respectively, which far exceed the recommended nutrient intakes (RNI) for salt (6g/day) and sodium (1.6g/day) (Bates, 2015). Of the dried seaweed products presented in **Table 2-4** and **Table 2-5**, *Laminaria digitata* and *Palmaria palmata* may have a favourable Na/K ratio for application as salt replacing condiments (1.03 and 0.84, respectively) (Rodrigues et al., 2015). However, low portion sizes of seaweed may be required to prevent excessive salt intake, given that a 5g daily portion of *Laminaria digitata* can provide up to 0.35g salt and 0.26g sodium, whilst *Palmaria palmata* may provide up to 0.27g salt and 0.15g sodium. These exceed salt and sodium levels in an equivalent amount of bacon (0.144g salt and 0.0575g sodium), which is regarded as a high salt food (Food Standards Agency, 2017).

2.4.4 Iodine

Iodine is a trace element required for the synthesis and function of triiodothyronine (T3) and thyroxine (T4) thyroid hormones. In Japan, where approximately 20 different types of seaweed are consumed, the majority being Wakame (*Undaria spp.*), Kombu (*Laminaria spp.*) and Nori (*Porphyra spp.*), iodine intake varies from 0.1 – 20mg/day (average intake = 1-3mg/day), which can exceed the upper tolerable limits of 600µg/day (EFSA) and 1100 µg/day (WHO) (EFSA, 2014; WHO, 2011; Zava and Zava, 2011)

The epidemiological evidence detailing the risks and benefits of iodine intake from seaweeds remains inconclusive. Seaweed consumption was associated with the increased risk of papillary carcinoma of the thyroid in postmenopausal Japanese women but not premenopausal women, whereas no association was established between seaweed consumption and total thyroid cancer risk or papillary carcinoma in premenopausal or postmenopausal women (Michikawa et al., 2012; Wang et al., 2016).

Iodine-induced hypothyroidism is reported in iodine-sufficient, kelp-consuming populations of Japan, and iodine-induced hyperthyroidism is also reported in individuals who consume kelp (Eliason, 1998; Konno et al., 1994; Mussig et al., 2006). The use of seaweed supplements is not recommended for pregnant women, owing to the variability and excessive iodine content of seaweeds, with particular concern for kelp based products (Bath et al., 2017; Zimmermann and Delange, 2004). Synergy between iodine supplementation and exposure to heavy metals in seaweed, such as mercury, may also impair thyroid function through the reduction of total T3 (Llop et al., 2015).

The iodine contents of the seaweeds presented in **Table 2-18**, **Table 2-19**, and **Table 2-20** range from 0.06mg/100g (*Ulva lactuca*) to 624.5mg/100g (*Laminaria digitata*), but many characterisation studies do not quantify iodine. Desideri *et al.* (Desideri et al., 2016) found that 3.3g of *Laminaria digitata* would provide 4017% of the tolerable daily intake for iodine, and suggested that habitual intake of seaweed with an iodine content >45mg/kg DW could impair thyroid function. Given that *Laminaria spp.* is widely abundant and currently used as a food ingredient, characterisation of iodine is warranted in *Laminaria* products owing to such high iodine content. In contrast, a 5g portion of *Porphyra tenera* is reported to provide only 80µg iodine (Teas et al., 2004).

Static *in vitro* digestion studies have reported seaweed iodine bioavailability in: *Laminaria spp.* (17 - 28%); *Sargassum fusiforme* (12%); *Palmaria palmata* (10%); *Undaria pinnatifida* (2 - 12%); *Himanthalia elongata* (4%); *Porphyra spp.* (5%); *Ulva rigida* (2%); *Cooked Himanthalia elongate* and *Sacchoriza polyschides* (<LOD) (Dominguez-Gonzalez et al., 2017; Nitschke and Stengel, 2015). Boiling has been shown to reduce the iodine content of *Alaria esculenta* (670 → 165 µg/g)*, Palmaria palmata* (97 → 66 µg/g) and *Ulva intestinalis* (92 → 79 µg/g), which may be beneficial cooking instructions for industry to provide to the consumer (Nitschke and Stengel, 2016).

In humans, urinary iodine excretion following *Ascophyllum nodosum* ingestion was reported as only 33% (potassium iodide control = 59%), where reduced iodine bioavailability was attributed to reduced release from the seaweed food matrix (i.e. bound to proteins, polysaccharides, polyphenols and pigments) (Hou et al., 2000). In a Caco-2 and HT29-MTX co-culture, iodine uptake following an *in vitro*

digestion was only 4 – 6% (Hijiki), 2 – 4% (Kombu), 4 – 7% (Wakame), which also suggests limited liberation of iodine species, limited solubility, or limited absorption (Dominguez-Gonzalez et al., 2017; Nathans et al., 1960; Nicola et al., 2012, 2009).

Urinary excretion of iodine from *Gracilaria verrucosa* and *Laminaria hyperborea* were reported as 101% and 90%, respectively in an iodine sufficient population, yet 85% and 61.5% in an iodine deficient population (Aquaron et al., 2002). Reduced urinary iodine excretion in the deficient cohort was attributed to increased iodine storage in the thyroid, thus, seaweed consumption may improve iodine status in those at risk of iodine deficiency, as demonstrated in a vegan population (Krajcovicova-Kudlackova et al., 2003; Lightowler and Davies, 1998; Remer et al., 1999).

Iodine absorption from *Laminaria japonica* is estimated as 57-71%, however, and serum TSH was significantly increased above the normal limits in 4/6 participants who consumed 15g/day of *Laminaria japonica* for 7-10 days, in 4/14 consuming 30g/day for 7-10 days, and 1/3 consuming 15g/day for 55-87 days, which corroborates previous evidence that kelp supplementation increased serum TSH over 4 weeks (Clark et al., 2003; Miyai et al., 2008). Urinary iodide excretion increased 30-fold and 44-fold from baseline (15g and 30g subgroups, respectively), but returned 7– 40 days after seaweed consumption ceased. Furthermore, iodine intake was improved without compromising thyroid function when 500mg *of Ascophyllum nodosum* containing 356µg iodine was given to healthy females for 14 days (Combet et al., 2014). Urinary iodine concentrations significantly increased, reflecting sufficient intake and subsequent renal excretion. Plasma concentrations

of T3, T4 free T3, or free T4 were unchanged between pre- and post-intervention, whilst serum TSH significantly increased, albeit within the normal range.

It is suggested that future human intervention studies should quantify iodine content of a seaweed food ingredient/supplement during product development, and urinary iodine concentration could be measured as a biomarker for iodine intake and bioavailability at time points throughout interventions (Katagiri et al., 2016; Nitschke and Stengel, 2015).

Efforts to disclose iodine contents on product labelling and the provision of cooking instructions represent strategies to prevent excessive iodine intake from seaweed food products. It is reported that only 22 of 224 seaweed-containing food products on sale in the UK disclosed iodine concentrations on their food labelling, while a total of 40 presented an estimate (Bouga and Combet, 2015). Some 26 products had the potential to exceed 600μg/day upper limits when serving suggestions were applied, but for the remaining 162, this was unknown. Guidance relating to an individuals' iodine status and how seaweed consumption may benefit the individual could also ensure consumer safety.

Variations in iodine concentration between seaweed species, season, and harvest location presents a challenge to the food industry, while there is limited and conflicting information as to how individual seaweeds may impact iodine status and thyroid health. Diligence regarding the long-term effects of seaweed consumption upon iodine status is needed, especially in non-consumers who wish to include seaweed into their diet.

2.4.6 Heavy Metals

A concern regarding seaweed consumption is exposure to heavy metals such as arsenic, aluminium, cadmium, lead, rubidium, silicon, strontium, and tin (Desideri et al., 2016). The contamination of seaweeds with heavy metals depends on habitat ecology, which has led to inconsistency in research findings. Seaweeds growing in contaminated sites, often a result of industry or poor sewage systems, accumulate heavy metals from the surrounding water and rocks, but seaweeds from contaminated sites have demonstrated little risk to human health due to heavy metal content (Phaneuf et al., 1999). Yet, perennial seaweeds suffer increased exposure to contaminants, and regular consumption may risk heavy metal toxicity in humans (Burger et al., 2012; Caliceti et al., 2002). The presence of arsenic, mercury, lead, and cadmium in 426 Korean dried seaweed products contained up to 0.2–6.7% of provisional tolerable weekly intakes when 8.5g seaweed was consumed per day (Hwang et al., 2010; Phaneuf et al., 1999). A concluding remark from this study called for continuous monitoring of heavy metals in seaweed-based food products, owing to differences in metal biosorption between species (Giusti, 2001; Jarvis and Bielmyer-Fraser, 2015; Murphy et al., 2007). This is evident in *Laminaria spp.* whereby, 3.3–12.5 g/day of *Laminaria digitata* is reported to contain 24 – 90µg cadmium, which corresponds to 40 – 150% of tolerable daily intake, while *Laminaria japonica* is reported to contain 0.45 – 0.80 mg/kg, which exceeds maximum limits for seaweed product legislation in France (0.5 mg/kg DW) and Australia/New Zealand (0.2 mg/kg DW), but not China (1.0 mg/kg) (Desideri et al., 2016; Zhao et al., 2012). **Table 2-18**, **Table 2-19** and **Table 2-20** provide further details of the heavy metal content of several brown, red and green seaweeds, respectively, albeit there is limited information regarding the

toxicokinetics of heavy metals ingested from seaweeds to understand potential health risks.

In a cross-sectional study of heavy metal concentrations in 3404 healthy Korean adults, urinary arsenic concentrations were significantly increased in both the second and third tertile for seaweed consumption (Park and Lee, 2013). Arsenic species were not determined in this study, whilst blood mercury was significantly higher in the highest consumers of seaweed versus the lowest consumer. Preliminary research shows that increased water temperatures can increase mercury absorption by fish, and the same biosorption of mercury may happen in seaweed (Pack et al., 2014). Considering global warming, trends in seaweed heavy metal content should also be monitored, in addition to the presence of rare earth elements, recently identified in northwest Mediterranean seaweeds (Squadrone et al., 2017). Ultimately, the extraction of bioactive or nutritional components from seaweeds may mitigate excessive heavy metal ingestion, which can be mutagenic and carcinogenic to humans, whilst the placental transfer of heavy metals from the mother to the foetus can cause neurological, developmental, and endocrine disorders in infants (Caserta et al., 2013; Koedrith et al., 2013; Taylor et al., 2014).

2.4.7 Arsenic

A major consideration for the exploitation of seaweeds as health foods or functional food ingredients is the need to speciate and quantify the levels of arsenic present in seaweed products. Arsenic species may be categorised as toxic (inorganic arsenic, iAs - class I carcinogen), non-toxic (arsenobetaine), and potentially toxic (fat-soluble arsenic, arsenosugars, and other organoarsenicals) (Feldmann and Krupp, 2011). The health risks pertained to inorganic hydrogen

arsenate species concern DNA damage, which predispose carcinogenesis, and consumption of iAs has been shown to increase the incidence of lung, bladder, skin, and kidney cancer and has also been linked with skin lesions, cardiovascular disease, neurological effects, and diabetes (Arslan et al., 2017; Khan et al., 2017; Taylor et al., 2017).

Most arsenic species in seaweeds are arsenosugars, typically ligated to glycerol, sulphonate or phosphonate. Arsenosugars resist degradation in the stomach, and upon entering the lower gastrointestinal tract, they are metabolised to at least 12 different metabolites, including dimethylarsinate (DMA), methylarsinate (MA), and dimethylarsinoylethanol (DMAE), but the toxicology of these metabolites is unknown (Andrewes et al., 2004; Feldmann and Krupp, 2011; Francesconi et al., 2002; Van Hulle et al., 2004).

Speciation and concentration of arsenic in brown seaweeds (*Ascophyllum nodosum, Laminaria digitata, Fucus vesiculosus, Fucus spiralis, Alaria esculenta*, and *Saccharina latissima*); red seaweeds (*Porphyra umbilicalus, Chondrus crispus, Gracliaria vermiculophylla,* and *Palmaria palmata)*; and green seaweeds (*Ulva prolifera* and *Ulva lactuca*) revealed that total arsenic ranged from 4.1 – 111 µg/g, with the majority of arsenic present as arsenosugars (iAs was <1.0 µg/g). (Caserta et al., 2013; Ronan et al., 2017). The exception was *Laminaria digitata*, which contained 2.8-20 µg/g iAs (USA) and 2.2 – 87 µg/g iAs (Ireland), which represents a large proportion of the total arsenic contents of *Laminaria digitata*, reported to range from 36 – 131 µg/g DW (Desideri et al., 2016; Ronan et al., 2017; Taylor and Jackson, 2016). In contrast, *Laminaria japonica* sourced from China contained 0.16 – 0.58 mg/kg iAs, which is below maximum limits set by China (1.0 mg/kg DW),

France (3 mg/kg DW), and Australia/New Zealand (1 mg/kg DW) (Food Standards Australia New Zealand, 2013; French Food Safety Agency, 2009; Zhao et al., 2012). Such variation in iAs contents may warrant regular testing for inorganic arsenic within *Laminaria spp.* food products.

Another recent study reported that iAs content (µg/g) was negligible in 23 seaweed food products except for: Hijiki (19.83); Agar (0.06); Nori (0.03), where total arsenic (µg/g) was: Hijiki (83.7) > Kombu (51.2) > Kelp Seasoning (43.5) > Arame (41.6) > Wakame (34.7) > Dried Red Seaweed (35.2) > Nori (19.4) > Dulse (12.1) > Agar (0.23) > Kelp Noodles (0.08) (Taylor et al., 2017). The amount of iAs within 112 edible seaweed products sold in Spain was also within safe limits, with the exception of Hijiki, where iAs ranged from 41.6 to117 µg/g (Almela et al., 2006).

Inclusion of 3% Hijiki powder has caused arsenic poisoning in rats, and there are current recommendations against the consumption of Hijiki in Asia, Australia, Europe and the USA, owing to its public health risk (Food Safety Authority of Ireland, 2015; Food Standards Agency, 2004; Food Standards Australia New Zealand, 2016; Government of Hong Kong Centre for Food Safety, 2011; Superior Health Council of Belgium, 2015; Yokoi and Konomi, 2012). Seaweeds such as Arame, Wakame, Kombu, and Nori, however, are suggested as safe products to eat because they contain <0.3 µg/g iAs, which is encouraging for food ingredient applications (Rose et al., 2007).

The amount of potentially bioaccessible arsenite (As III), arsenate (As V), methylarsonate (MMA), and dimethylarsinate (DMA) following the *in vitro* digestion of *Laminaria japonica*, *Undaria pinnatifida*, *Hizikia fusiformis*, *Porphyra yezoensis*, and *Enteromorpha prolifera* was low enough to indicate no hazard of

inorganic arsenic to human health (Zhao et al., 2014). However, the cooking process has been shown to increase iAs species within *Porphyra spp.* and *Hijika fusiforme*, after baking and boiling, respectively, whilst soaking, cooking, boiling, and washing/soaking seaweeds reduced total arsenic by 58.8%, 91.5%, 50%, and 60% respectively (Hajeb et al., 2014; Laparra et al., 2003).

Studies investigating the bioavailability of arsenic from *Porphyra spp.* have been carried out, where Wei *et al*. (Wei et al., 2003) reported that total urinary As peaked (average = 92.5 ng/ml), after 20 – 30 hours, resulting in a 20 fold increase in DMA, before returning to normal levels after 80 hours. Another study showed that arsenic metabolites, DMA and 2-dimethylarsinoyl ethanol (DMAE), were detected in urine of 5 volunteers whom consumed 20 – 25g of *Laminaria spp.* (total As = 43.2 μg/g), where peak arsenic/creatinine ratio was 228, 158, 141, 72, and 70 ng/ml and levels normalised after 80 hours (Van Hulle et al., 2004).

Consumption of 10g/day Nori, Kombu or Wakame for 3 days, followed by a 3-day washout between seaweeds, demonstrated increased arsenosugars, DMA, thio-DMAA, and thio-DMAE in 24h urine samples following seaweed consumption, which varied between seaweeds and individuals (Taylor et al., 2017). Toxic thio-DMA was only present at trace levels, and the authors identified thio-DMAE and thio-DMAA as unique arsenosugar metabolites and suggested their use as a urinary biomarker for dietary intake of arsenic from seaweeds.

The arsenosugars present in seaweed are demonstrated to resist cooking and *in vitro* digestion processes, and have been suggested, in part, to be absorbed into the hepatic portal system intact (Almela et al., 2005). Human studies have shown considerable differences in the rate of excretion of arsenosugars ranging from 4-

95% (Taylor et al., 2017). The high variability associated with arsenosugar metabolism may be attributable to interpersonal differences in endogenous digestion, gut microbiota composition and activity, passage across the intestinal barrier, or transformation in the liver (Taylor et al., 2017). Thus, there is a need to characterise the metabolic fate of arsenosugars in order to clarify the safety associated with arsenosugar rich seafoods (Mania et al., 2015).

Whilst there are efforts by regulatory bodies to provide guidance in relation to arsenic intake, for example, the UK Food Standards Agency has advice to avoid against consuming *Sargassum fusiforme* (Hijiki), owing to significant food safety concerns over high levels of iAs, there is a need for clearer regulation and guidance in the permissible arsenic content of foods (Food Standards Agency, 2004). Regular environmental assessment and analysis of the arsenic species present in seaweed-containing food products may be required to ascertain exposure and potential toxicity of heavy metals to alleviate health risks (Food Standards Australia New Zealand, 2013; Hwang et al., 2010). Indirect exposure to arsenic could also be a factor if seaweed is included in farming practises (Adamse et al., 2017; Castlehouse et al., 2003). Despite this, the majority of edible seaweeds have been reported to contain heavy metals in safe amounts. As with iodine, it is suggested that food regulation should ensure the disclosure of heavy metal contents on food labelling, with legal limits for seaweed iAs (Brandon et al., 2014). Cooking methods and food processing procedures may help to reduce the amount of heavy metal present in edible seaweeds, but the greatest challenge to the regulatory bodies in developing safe limits is the inter-individual differences in biotransformation, metabolism, and excretion. Whilst the greatest challenge to industry is the high within-species

variability of arsenic in the seaweed, and the potential costs of regularly monitoring product(s).

2.5 Whole seaweeds: Impact of habitual intake on health

Whilst this review highlights the lack of human intervention trials investigating the potential risks and benefits of consuming seaweed components, some observational evidence does exist. Epidemiological evidence indicates that seaweed-containing diets are inversely associated with all-cause mortality and cardiovascular disease mortality in Japanese adults; yet, Korean men with metabolic syndrome are reported to consume significantly more seaweed than those without metabolic syndrome, although no mechanistic insight was disseminated (Nanri et al., 2017; Shin et al., 2009).

Consumption of *Porphyra spp*. was inversely associated with breast cancer risk in premenopausal women, but not postmenopausal women, and no association was found between *Undaria pinnatifida* consumption and breast cancer risk (Key et al., 2010; Teas et al., 2011; Y. J. Yang et al., 2010). A study by Michikawa *et al.* (Michikawa et al., 2012) identified a positive association between seaweed consumption and the risk of thyroid cancer (especially papillary carcinoma) in postmenopausal women, whilst Wang *et al.* (Wang et al., 2016) did not find an association between seaweed intake and thyroid cancer incidence in premenopausal or in postmenopausal women. A case-controlled study by Hoshiyama et al. (Hoshiyama et al., 1993; Hoshiyama and Sasaba, 1992) implicated an inverse relationship of seaweed with stomach and colon cancer, nevertheless interpretation warrants caution in light of the low sample power of the studies.

An inverse association is also reported between *Undaria pinnatifida*, *Sargassum fusiforme*, and *Porphyra spp.* intake and allergic rhinitis prevalence in pregnant Japanese women (n = 1002) (Miyake et al., 2006). The study did not measure iodine intake or iodine status, which could have contributed to the knowledge of iodine intake from seaweeds during pregnancy, where current recommendations in Australia and New Zealand limit brown seaweed intake to one portion per week in pregnant women (Food Standards Australia New Zealand, 2011). There are also concerns regarding the potential for seaweed to contribute to food borne infections as noted by reports of Norovirus contamination of *Enteromorpha spp.*, and the presence of polycavernoside A toxin in *Gracilaria edulis* (Haddock and Cruz, 1991; Park et al., 2015; Yotsu-Yamashita et al., 2004).

Current Asian populations are reported to consume less seaweed than previous generations, shifting towards a high energy and low fibre westernised diet, which promotes the development of metabolic syndrome, and has increased the number of iodine deficient individuals in Japan (Katagiri et al., 2015; Zhou et al., 2003). One recent intervention study in a European population concluded that *Palmaria palmata* consumption could improve iodine status in adults, where serum thyroid stimulating hormone (TSH) was significantly increased (within the normal clinical range) following 5g/day *Palmaria palmata* for 28 days (Allsopp et al., 2016). The authors of this study highlighted the need to characterise seaweed composition when undertaking human interventions to help ascertain which components of seaweed impact on health, immune function and disease risk. Whilst the evidence from observational studies discussed above may provide indications of potential benefits, the outcomes must be treated with considerable caution and attempts

made to verify these observations using randomised controlled trials with suitable biomarkers, as well as supportive *in vitro* and *in vivo* animal studies to elucidate the mechanisms of action.

2.6 Conclusion

Edible seaweeds are a rich and sustainable source of macronutrients (particularly dietary fibre) and micronutrients, but if seaweeds shall contribute towards future global food security, legislative measures to ensure monitoring and labelling of food products are needed to safeguard against excessive intakes of salt, iodine and heavy metals.

Whilst the heavy metal concentration in edible seaweeds is generally below toxic levels, bioaccumulation of arsenic is a risk, and more studies concerning heavy metal toxicokinetics are needed. A trade-off between iodine and/or heavy metal ingestion and the amount of whole seaweed needed to obtain meaningful amounts of PUFAs, protein, or dietary fibre may limit portion size. Therefore, the extraction of individual components from the complex seaweed matrix is a legitimate strategy to create value-added products, where the efficacy of novel bioactive components extracted from seaweeds are increasingly studied as agents to combat non-communicable diseases.

Looking ahead, more human intervention studies are needed to establish how chronic consumption of whole seaweeds and their extracted bioactive components impact human health, with defined health-related end points. Mechanisms of action must also be elucidated to substantiate any future claims associated with

seaweed consumption and human health benefits, to suffice application within food and nutraceutical industries.

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2.8 Tables

Table 2-1 Macronutrient content of brown seaweeds

Reference	Seaweed	Country	Date harvested	Protein (%)	Percent RNI supplied by 5-g portion	Lipid (%)	Percent RNI supplied by 5-g portion	Carbohydrate (%)	Percent RNI supplied by 5-g portion	Fiber (%)	Percent RNI supplied by 5-g portion
Maehre et al (2014)[19]	*Alaria esculenta*	Norway	May 2010	9.11	0.91	1.40	0.10	–	–	–	–
Schiener et al (2015)[17]	*Alaria esculenta*	Scotland	Mar 2011 – Jul 2011	11.04	1.10	–	–	72.10	1.39	–	–
Tabarsa et al (2012)[20]	*Colpomenia sinuosa*	Iran	Apr 2008	10.11	1.01	1.46	0.10	–	–	9.50	1.58
El-Said & El-Sikaily (2013)[21]	*Colpomenia sinuosa*	Egypt	Apr 2011	–	–	–	–	11.80	0.23	–	–
Rohani-Ghadikolaei et al (2012)[22]	*Colpomenia sinuosa*	Iran	–	9.20	0.92	1.50	0.11	32.10	0.62	–	–
Tabarsa et al (2012)[20]	*Dictyota dichotoma*	Iran	Apr 2008	17.73	1.77	2.94	0.21	–	–	10.50	1.75
Smith et al (2010)[23]	*Durvillaea antarctica*	New Zealand	Apr 2004	7.26	0.73	2.03	0.15	58.82	1.13	–	–
Ortiz et al (2006)[24]	*Durvillaea antarctica*	Chile	Nov 2013	10.40	1.04	0.80	0.06	70.90	1.36	71.40	11.90
Ortiz et al (2006)[24]	*Durvillaea antarctica*	Chile	Nov 2013	11.60	1.16	4.30	0.31	58.40	1.12	56.40	9.40
Smith et al (2010)[23]	*Ecklonia radiata*	New Zealand	Aug 2004	9.60	0.96	1.80	0.13	66.90	1.29	–	–
Smith et al (2010)[23]	*Ecklonia* spp	New Zealand	–	9.78	0.98	0.80	0.06	69.61	1.34	–	–
Mišurcová et al (2010)[25]	*Eisenia bicyclis*	Japan	–	–	–	–	–	–	–	11.15	1.86
Paiva et al (2014)[26]	*Fucus spiralis*	Portugal	Jan 2013	9.71	0.97	5.23	0.37	17.59	0.34	–	–
Marsham et al (2007)[27]	*Fucus serratus*	United Kingdom	–	17.40	1.74	1.80	0.13	–	–	26.20	4.37
Maehre et al (2014)[19]	*Fucus vesiculosus*	Norway	May 2010	6.11	0.61	3.08	0.22	–	–	–	–
Sanchez-Machado et al (2004)[28]	*Himanthalia elongata*	Spain	–	5.46	0.55	0.97	0.07	–	–	–	–
Sanchez-Machado et al (2004)[28]	*Himanthalia elongata*	Spain	–	10.95	1.10	0.93	0.07	–	–	–	–
Mišurcová et al (2010)[25]	*Hizikia fusiformis*	Japan	–	–	–	–	–	–	–	17.52	2.92
Smith et al (2010)[25]	*Hormosira banksii*	New Zealand	Apr 2004	6.07	0.61	2.63	0.19	62.90	1.21	–	–
Maehre et al (2014)[19]	*Laminaria digitata*	Norway	May 2010	5.31	0.53	0.99	0.07	–	–	–	–
Schiener et al (2015)[17]	*Laminaria digitata*	Scotland	Aug 2010 – Oct 2011	6.90	0.69	–	–	70.70	1.36	–	–
Marsham et al (2007)[27]	*Laminaria digitata*	United Kingdom	–	15.90	1.59	0.50	0.04	–	–	16.60	2.77
Maehre et al (2014)[19]	*Laminaria hyperborea*	Norway	May 2010	5.02	0.50	1.28	0.09	–	–	–	–
Schiener et al (2015)[17]	*Laminaria hyperborea*	Scotland	Aug 2010 – Oct 2011	6.80	0.68	–	–	65.50	1.26	–	–
Mišurcová et al (2010)[25]	*Laminaria japonica*	Japan	–	–	–	–	–	–	–	10.45	1.74
Sanchez-Machado et al (2004)[28]	*Laminaria ochroleuca*	Spain	–	7.49	0.75	0.92	0.07	–	–	–	–
Smith et al (2010)[25]	*Macrocystis* spp	New Zealand	–	11.02	1.10	1.56	0.11	44.54	0.86	–	–
Tabarsa et al (2012)[20]	*Padina pavonica*	Iran	Apr 2008	11.83	1.18	1.79	0.13	–	–	11.00	1.83
Maehre et al (2014)[19]	*Petvetia canaliculata*	Norway	May 2010	5.72	0.57	4.78	0.34	–	–	–	–
Schiener et al (2015)[17]	*Saccharina latissima*	Scotland	Aug 2010 – Oct 2011	7.10	0.71	–	–	63.10	1.21	–	–
Sanchez-Machado et al (2004)[28]	*Saccorhiza polyschides*	Spain	–	13.10	1.31	0.70	0.05	–	–	–	–
Rodrigues et al (2015)[29]	*Saccorhiza polyschides*	Portugal	Apr 2012	14.44	1.44	1.10	0.08	45.60	0.88	–	–
Rohani-Ghadikolaei et al (2012)[22]	*Sargassum ilicifolium*	Iran	–	8.90	0.89	2.00	0.14	32.90	0.63	–	–
Matanjun et al (2009)[30]	*Sargassum polycystum*	Borneo	–	5.40	0.54	0.29	0.02	33.49	0.64	39.67	6.61
Marinho-Soriano et al (2006)[31]	*Sargassum vulgare*	Brazil	Jul 2000 – Jun 2001	13.61	1.36	0.49	0.04	61.60	1.18	7.74	1.29
El-Said & El-Sikaily (2013)[21]	*Sargassum linifolium*	Egypt	Apr 2011	–	–	–	–	8.59	0.17	–	–
Rodrigues et al (2015)[29]	*Sargassum muticum*	Portugal	Apr 2012	16.90	1.69	1.45	0.10	49.30	0.95	–	–
Peng et al (2013)[32]	*Sargassum naozhouense*	China	Jul 2011	11.20	1.12	1.06	0.08	47.43	0.91	4.83	0.81
Smith et al (2010)[23]	*Undaria pinnatifida*	New Zealand	–	14.21	1.42	3.13	0.22	45.08	0.87	–	–
Sanchez-Machado et al (2004)[28]	*Undaria pinnatifida*	Spain	–	18.00	1.80	1.05	0.08	–	–	–	–
Smith et al (2010)[23]	*Undaria pinnatifida*	New Zealand	Apr – Sep 2004	19.66	1.97	3.30	0.24	50.40	0.97	–	–
Mišurcová et al (2010)[25]	*Undaria pinnatifida*	Japan	–	–	–	–	–	–	–	9.03	1.51
Mišurcová et al (2010)[25]	*Undaria pinnatifida*	Japan	–	–	–	–	–	–	–	15.53	2.59

Abbreviation: RNI, Reference Nutrient Intake.

Table 2-2. Macronutrient content of red seaweeds

Reference	Seaweed	Country	Date harvested	Protein (%)	Percent RNI supplied by 5-g portion	Lipid (%)	Percent RNI supplied by 5-g portion	Carbohydrate (%)	Percent RNI supplied by 5-g portion	Fiber (%)	Percent RNI supplied by 5-g portion
Parjikolaei et al (2016)[33]	*Ahnfeltia plicata*	Denmark	Sep 2011	31.10	3.11	1.10	0.08	59.10	1.14	–	–
Marsham et al (2007)[27]	*Ceramium* spp	United Kingdom	–	31.20	3.12	0.60	0.04	–	–	33.70	5.62
Parjikolaei et al (2016)[33]	*Chondrus crispus*	Denmark	Sep 2011	26.40	2.64	1.00	0.07	53.30	1.03	–	–
Marsham et al (2007)[27]	*Corallina officinalis*	United Kingdom	–	6.90	0.69	0.30	0.02	–	–	9.40	1.57
Parjikolaei et al (2016)[33]	*Delesseria sanguinea*	Denmark	Sep 2011	23.40	2.34	1.20	0.09	51.20	0.98	–	–
Parjikolaei et al (2016)[33]	*Dilsea carnosa*	Denmark	Sep 2011	21.50	2.15	1.20	0.09	53.00	1.02	–	–
Marsham et al (2007)[27]	*Dumontia contorta*	United Kingdom	–	31.70	3.17	0.12	0.01	–	–	34.30	5.72
Matanjun et al (2009)[30]	*Eucheuma cottonii*	Borneo	–	9.76	0.98	1.10	0.08	26.49	0.51	25.05	4.18
Parjikolaei et al (2016)[33]	*Furcellaria lumbricalis*	Denmark	Sep 2011	20.60	2.06	1.00	0.07	55.40	1.07	–	–
Paiva et al (2014)[26]	*Gelidium microdon*	Azores (Portugal)	Jan 2013	–	–	2.44	0.17	–	–	–	–
Marinho-Soriano et al (2006)[31]	*Gracilaria cervicornis*	Brazil	Jul 2000 – Jun 2001	19.70	1.97	0.43	0.03	63.10	1.21	5.65	0.94
El-Said & El-Sikaily (2013)[21]	*Gracilaria compressa*	Egypt	Apr 2011	–	–	–	–	11.62	0.22	–	–
Rohani-Ghadikolaei et al (2012)[22]	*Gracilaria corticata*	Iran	–	19.30	1.93	1.80	0.13	43.00	0.83	–	–
Sakthivel & Devi (2015)[34]	*Gracilaria edulis*	India	–	0.67	0.07	0.83	0.06	10.16	0.20	8.90	1.48
Francavilla et al (2013)[35]	*Gracilaria gracilis*	Italy	Jul 2011	31.00	3.10	1.98	0.14	27.50	0.53	–	–
Francavilla et al (2013)[35]	*Gracilaria gracilis*	Italy	Apr 2012	41.00	4.10	1.40	0.10	34.10	0.66	–	–
Francavilla et al (2013)[35]	*Gracilaria gracilis*	Italy	Oct 2011	41.00	4.10	1.38	0.10	24.80	0.48	–	–
Francavilla et al (2013)[35]	*Gracilaria gracilis*	Italy	Jan 2012	45.00	4.50	1.12	0.08	31.10	0.60	–	–
Rodrigues et al (2015)[29]	*Gracilaria gracilis*	Portugal	Apr 2012	20.20	2.02	0.60	0.04	46.60	0.90	–	–
Tabarsa et al (2012)[20]	*Gracilaria salicornia*	Iran	Apr 2008	9.58	0.96	2.00	0.14	–	–	10.40	1.73
Parjikolaei et al (2016)[33]	*Gracilaria vermiculophylla*	Denmark	Sep 2011	17.80	1.78	1.30	0.09	61.90	1.19	–	–
El-Said & El-Sikaily (2013)[21]	*Gracilaria verrucosa*	Egypt	Apr 2011	–	–	–	–	11.15	0.21	–	–
Rodrigues et al (2015)[29]	*Grateloupia turuturu*	Portugal	Apr 2012	22.50	2.25	2.20	0.16	43.20	0.83	–	–
Wong & Cheung (2000)[36]	*Hypnea charoides*	Hong Kong	Dec 1997	18.40	1.84	1.48	0.11	–	–	50.30	8.38
Wong & Cheung (2000)[36]	*Hypnea japonica*	Hong Kong	Dec 1997	19.00	1.90	1.42	0.10	–	–	53.20	8.87
Siddique (2013)[37]	*Hypnea musciformis*	Bangladesh	May 2005	18.64	1.86	1.27	0.09	20.60	0.40	37.92	6.32
El-Said & El-Sikaily (2013)[21]	*Hypnea musciformis*	Egypt	Apr 2011	–	–	–	–	11.17	0.21	–	–
Siddique (2013)[37]	*Hypnea pannosa*	Bangladesh	May 2005	16.31	1.63	1.56	0.11	22.89	0.44	40.59	6.77
Rohani-Ghadikolaei et al (2012)[22]	*Hypnea valentiae*	Iran	–	16.50	1.65	2.80	0.20	31.80	0.61	–	–
El-Said & El-Sikaily (2013)[21]	*Jania rubens*	Egypt	Apr 2011	–	–	–	–	12.96	0.25	–	–
Fayaz et al (2005)[38]	*Kappaphycus alvarezii*	India	–	16.24	1.62	0.74	0.05	27.40	0.53	29.40	4.90
Kumar et al (2015)[39]	*Kappaphycus alvarezii*	India	Sep 2004 – Apr 2006	19.25	1.93	0.64	0.05	–	–	14.52	2.42
Marsham et al (2007)[27]	*Mastocarpus stellatus*	United Kingdom	–	25.40	2.54	3.00	0.21	–	–	16.60	2.77
Gressler et al (2011)[40]	*Ochtodes secundiramea*	Brazil	–	10.10	1.01	3.54	0.25	45.07	0.87	–	–
Parjikolaei et al (2016)[33]	*Odonthalia dentata*	Denmark	Sep 2011	17.80	1.78	–	–	–	–	–	–
Paiva et al (2014)[26]	*Osmundea pinnatifida*	Portugal	Jan 2013	20.79	2.08	7.53	0.54	17.61	0.34	–	–

(continued)

Table 2-2 continued. Macronutrient content of red seaweeds

Reference	Seaweed	Country	Date harvested	Protein (%)	Percent RNI supplied by 5-g portion	Lipid (%)	Percent RNI supplied by 5-g portion	Carbohydrate (%)	Percent RNI supplied by 5-g portion	Fiber (%)	Percent RNI supplied by 5-g portion
Rodrigues et al (2015)[29]	*Osmundea pinnatifida*	Portugal	Apr 2012	23.80	2.38	0.90	0.06	32.40	0.62	–	–
Marsham et al (2007)[27]	*Osmundea pinnatifida*	United Kingdom	–	27.30	2.73	4.30	0.31	–	–	25.60	4.27
Maehre et al (2014)[19]	*Palmaria palmata*	Norway	Jun 2012	12.26	1.23	1.36	0.10	–	–	–	–
Parjikolaei et al (2016)[33]	*Palmaria palmata*	Denmark	Sep 2011	14.90	1.49	1.20	0.09	71.00	1.37	–	–
Mišurcová et al (2010)[25]	*Palmaria palmata*	United States	–	–	–	–	–	–	–	5.05	0.84
Sanchez-Machado et al (2004)[28]	*Palmaria* spp	Spain	–	13.87	1.39	1.80	0.13	–	–	–	–
Parjikolaei et al (2016)[33]	*Phycodrys rubens*	Denmark	Sep 2011	28.80	2.88	1.30	0.09	40.20	0.77	–	–
Gressler et al (2011)[40]	*Plocamium brasiliense*	Brazil	–	15.72	1.57	3.63	0.26	52.03	1.00	–	–
Marsham et al (2007)[27]	*Polysiphonia* spp	United Kingdom	–	31.80	3.18	0.05	0.00	–	–	52.80	8.80
Cian et al (2014)[41]	*Porphyra columbina*	Argentina	Aug – Oct 2010	24.61	2.46	0.25	0.02	–	–	48.02	8.00
Sanchez-Machado et al (2004)[28]	*Porphyra* spp	Spain	–	24.11	2.41	1.03	0.07	–	–	–	–
Paiva et al (2014)[26]	*Porphyra* spp	Portugal	Jan 2007	24.82	2.48	8.88	0.63	25.37	0.49	–	–
Smith et al (2010)[23]	*Porphyra* spp	New Zealand	–	26.36	2.64	3.03	0.22	43.99	0.85	–	–
Smith et al (2010)[23]	*Porphyra* spp	New Zealand	May – Oct 2004	32.71	3.27	2.00	0.14	45.40	0.87	–	–
Marsham et al (2007)[27]	*Porphyra* spp	United Kingdom	–	44.00	4.40	0.70	0.05	–	–	33.50	5.58
Cofrades et al (2010)[42]	*Porphyra umbilicalis*	Spain	–	39.00	3.90	–	–	–	–	–	–
El-Said & El-Sikaily (2013)[21]	*Pterocladia capillacea*	Egypt	Apr 2011	–	–	–	–	9.64	0.19	–	–
El-Said & El-Sikaily (2013)[21]	*Pterocladia capillacea*	Egypt	Apr 2011	–	–	–	–	11.20	0.22	–	–
El-Said & El-Sikaily (2013)[21]	*Pterocladia capillacea*	Egypt	Apr 2011	–	–	–	–	7.89	0.15	–	–
El-Said & El-Sikaily (2013)[21]	*Pterocladia capillacea*	Egypt	Apr 2011	–	–	–	–	8.06	0.16	–	–
Paiva et al (2014)[26]	*Pterocladia capillacea*	Azores (Portugal)	Jan 2013	–	–	4.32	0.31	–	–	–	–
Maehre et al (2014)[19]	*Vertebrata lanosa*	Norway	June 2012	11.56	1.16	1.55	0.11	–	–	–	–

Abbreviation: RNI, Reference Nutrient Intake.

Table 2-3. Macronutrient content of green seaweeds

Reference	Seaweed	Country	Date harvested	Protein (%)	Percent RNI supplied by 5-g portion	Lipid (%)	Percent RNI supplied by 5-g portion	Carbohydrate (%)	Percent RNI supplied by 5-g portion	Fiber (%)	Percent RNI supplied by 5-g portion
Nguyen et al (2011)[43]	*Caulerpa lentillifera*	Taiwan	–	9.26	0.93	1.57	0.11	64.00	1.23	2.97	0.50
Matanjun et al (2009)[30]	*Caulerpa lentillifera*	Borneo	–	10.41	1.04	1.11	0.08	38.66	0.74	32.99	5.50
Ratana-Arporn & Chirapart (2006)[44]	*Caulerpa lentillifera*	Thailand	March	12.49	1.25	0.86	0.06	59.27	1.14	3.17	0.53
Mandlik et al (2014)[45]	*Caulerpa racemosa*	India	–	–	–	–	–	10.02	0.19	–	–
Koklam & Vasuki (2013)[46]	*Caulerpa taxifolia*	India	–	12.44	1.24	0.32	0.02	23.86	0.46	–	–
Maehre et al (2014)[19]	*Cladophora rupestris*	Norway	May 2010	3.42	0.34	0.76	0.05	–	–	–	–
Marsham et al (2007)[27]	*Cladophora rupestris*	United Kingdom	–	29.80	2.98	1.00	0.07	–	–	45.70	7.62
Rodrigues et al (2015)[29]	*Codium tomentosum*	Portugal	April 2012	18.80	1.88	3.60	0.26	32.80	0.63	–	–
El-Said & El-Sikaily (2013)[21]	*Codium tomentosum*	Egypt	April 2011	–	–	–	–	11.15	0.21	–	–
Aguilera-Morales et al (2005)[47]	*Enteromorpha* spp	Mexico	Winter 1997	9.45	0.95	2.24	0.16	–	–	–	–
Aguilera-Morales et al (2005)[47]	*Enteromorpha* spp	Mexico	Winter 1998	14.10	1.41	2.27	0.16	–	–	–	–
Pirian et al (2016)[48]	*Ulva californica*	Iran	March 2015	15.20	1.52	3.75	0.27	–	–	–	–
Pirian et al (2016)[48]	*Ulva californica*	Iran	February 2015	15.76	1.58	3.45	0.25	–	–	–	–
Pirian et al (2016)[48]	*Ulva compressa*	Iran	February 2015	18.12	1.81	1.03	0.07	–	–	–	–
Pirian et al (2016)[48]	*Ulva compressa*	Iran	March 2015	18.64	1.86	0.90	0.06	–	–	–	–
Paiva et al (2016)[49]	*Ulva compressa*	Azores (Portugal)	April 2013	–	–	1.67	0.12	–	–	–	–
Pirian et al (2016)[48]	*Ulva fasciata*	Iran	March 2015	14.06	1.41	0.56	0.04	–	–	–	–
Pirian et al (2016)[48]	*Ulva fasciata*	Iran	February 2015	14.69	1.47	0.47	0.03	–	–	–	–
Pirian et al (2016)[48]	*Ulva flexuosa*	Iran	March 2015	10.55	1.06	2.82	0.20	–	–	–	–
Pirian et al (2016)[48]	*Ulva flexuosa*	Iran	March 2015	11.23	1.12	2.34	0.17	–	–	–	–
Escobido et al (2016)[50]	*Ulva intestinalis*	Philippines	January – April 2016	5.57	0.56	0.43	0.03	37.28	0.72	–	–
Rohani-Ghadikolaei et al (2012)[22]	*Ulva intestinalis*	Iran	–	10.50	1.05	2.90	0.21	35.50	0.68	–	–
Maehre et al (2014)[19]	*Ulva intestinalis*	Norway	May 2010	11.33	1.13	1.62	0.12	–	–	–	–
El-Said & El-Sikaily (2013)[21]	*Ulva intestinalis*	Egypt	April 2011	–	–	–	–	8.72	0.17	–	–
Wong & Cheung (2000)[36]	*Ulva lactuca*	Hong Kong	December 1997	7.06	0.71	1.64	0.12	–	–	55.40	9.23
Yaich et al (2011)[51]	*Ulva lactuca*	Tunisia	July 2007	8.46	0.85	7.87	0.56	–	–	54.90	9.15
Maehre et al (2014)[19]	*Ulva lactuca*	Norway	June 2012	8.65	0.87	2.00	0.14	–	–	–	–
Tabarsa et al (2012)[20]	*Ulva lactuca*	Iran	April 2008	10.69	1.07	0.99	0.07	–	–	5.60	0.93
Pirian et al (2016)[48]	*Ulva lactuca*	Iran	February 2015	20.85	2.09	0.85	0.06	–	–	–	–
Pirian et al (2016)[48]	*Ulva lactuca*	Iran	March 2015	21.55	2.16	0.75	0.05	–	–	–	–
Bikker et al (2016)[52]	*Ulva lactuca*	Ireland	May 2015	26.30	2.63	2.11	0.15	24.00	0.46	–	–
Ortiz et al (2006)[24]	*Ulva lactuca*	Chile	November 2013	27.20	2.72	0.30	0.02	61.50	1.18	60.50	10.08
Marsham et al (2007)[27]	*Ulva lactuca*	United Kingdom	–	29.00	2.90	0.50	0.04	–	–	32.90	5.48
El-Said & El-Sikaily (2013)[21]	*Ulva lactuca*	Egypt	April 2011	–	–	–	–	11.15	0.21	–	–
El-Said & El-Sikaily (2013)[21]	*Ulva lactuca*	Egypt	April 2011	–	–	–	–	10.95	0.21	–	–
El-Said & El-Sikaily (2013)[21]	*Ulva lactuca*	Egypt	April 2011	–	–	–	–	11.54	0.22	–	–
El-Said & El-Sikaily (2013)[21]	*Ulva lactuca*	Egypt	April 2011	–	–	–	–	11.28	0.22	–	–

(continued)

Table 2-3 continued. Macronutrient content of green seaweeds

Reference	Seaweed	Country	Date harvested	Protein (%)	Percent RNI supplied by 5-g portion	Lipid (%)	Percent RNI supplied by 5-g portion	Carbohydrate (%)	Percent RNI supplied by 5-g portion	Fiber (%)	Percent RNI supplied by 5-g portion
El-Said & El-Sikaily (2013)[21]	*Ulva lactuca*	Egypt	April 2011	–	–	–	–	11.41	0.22	–	–
El-Said & El-Sikaily (2013)[21]	*Ulva lactuca*	Egypt	April 2011	–	–	–	–	11.15	0.21	–	–
El-Said & El-Sikaily (2013)[21]	*Ulva lactuca*	Egypt	April 2011	–	–	–	–	10.16	0.20	–	–
Rohani-Ghadikolaei et al (2012)[22]	*Ulva lactuca*	Iran	–	17.10	1.71	3.60	0.26	59.10	1.14	–	–
Pirian et al (2016)[48]	*Ulva linza*	Iran	March 2015	10.16	1.02	3.70	0.26	–	–	–	–
Pirian et al (2016)[48]	*Ulva linza*	Iran	March 2015	10.45	1.05	3.30	0.24	–	–	–	–
Pirian et al (2016)[48]	*Ulva prolifera*	Iran	February 2015	19.87	1.99	6.06	0.43	–	–	–	–
Pirian et al (2016)[48]	*Ulva prolifera*	Iran	March 2015	20.30	2.03	6.06	0.43	–	–	–	–
Ratana-Arporn & Chirapart (2006)[44]	*Ulva reticulata*	Thailand	May	21.06	2.11	0.75	0.05	55.77	1.07	4.84	0.81
Taboada et al (2010)[53]	*Ulva rigida*	Spain	–	17.80	1.78	0.90	0.06	42.60	0.82	11.90	1.98
Paiva et al (2014)[26]	*Ulva rigida*	Azores (Portugal)	April 2013	–	–	1.02	0.07	–	–	–	–
Smith et al (2010)[23]	*Ulva stenophylla*	New Zealand	April 2004	20.43	2.04	1.24	0.09	55.60	1.07	–	–

Abbreviation: RNI, Reference Nutrient Intake.

Table 2-4. Nutritional information for a selection of seaweed products sold throughout the UK and the Republic of Ireland.

	%RNI Per 5g Portion							
Seaweed Product	Energy (Kcal)	Fat	Saturated Fat	Carbohydrate	Sugar	Protein	Salt	Dietary Fibre
Brown Seaweed								
Alaria esculenta	0.21	0.10	0.18	0.03	0.01	1.30	3.75	6.52
Alaria esculenta **	0.86	0.09	-	0.88	-	2.00	-	-
Ascophyllum nodosum[1]	-	-	-	0.43	-	0.24	-	3.33
Fucus vesiculosus	0.21	0.05	0.08	0.15	0.07	1.00	0.11	4.67
Himanthalia elongata	0.24	0.05	0.08	0.15	0.08	1.00	4.75	5.25
Himanthalia elongata	0.24	0.05	0.08	0.15	0.08	1.00	4.75	5.25
Laminaria digitata	0.24	0.14	0.23	0.04	0.01	1.40	3.67	5.35
Laminaria digitata	0.31	0.14	0.23	0.06	0.00	1.42	3.83	4.92
Laminaria digitata **	0.75	0.07	-	0.92	-	1.40	-	-
Laminaria spp.	0.69	0.03	0.03	0.86	0.01	1.18	5.83	3.68
Saccharina latissima	0.24	0.05	0.08	0.15	0.10	1.00	3.75	5.27
Saccharina latissima **	0.51	0.04	-	1.17	-	1.10	-	-
Red Seaweed								
Chondrus crispus	0.16	0.09	0.03	0.04	0.01	1.00	1.08	8.05
Chondrus crispus	0.33	0.10	-	0.03	0.00	1.12	0.33	7.95
Chondrus crispus **	0.86	0.21	-	1.27	-	1.80	-	-
Palmaria palmata	0.80	0.18	0.25	0.15	0.71	1.48	4.48	7.95
Palmaria palmata	0.24	0.11	0.08	0.03	0.01	2.00	3.00	6.23
Palmaria palmata	0.59	0.11	0.10	0.44	0.06	1.38	4.08	6.15
Palmaria palmata **	0.71	0.21	-	0.96	-	2.10	-	-
Porphyra spp.	0.27	0.09	0.15	0.04	0.03	1.50	0.58	-
Porphyra spp. **	0.99	0.18	-	1.46	-	3.70	-	-

*Calculated from Dietary Reference Values for Energy (Scientific Advisory Committee on Nutrition, 2015). ** Maximum values taken from a range.

[1] Reported as %RNI for a 2g serving suggestion. [2] Reported as %RNI for a 1g serving suggestion (ingredient) and recommended dose (2x 500mg capsules per day - supplement).

Table 2-4 continued. Nutritional information for a selection of seaweed products sold throughout the UK and the Republic of Ireland.

	%RNI Per 5g Portion							
Seaweed Product	Energy (Kcal)	Fat	Saturated Fat	Carbohydrate	Sugar	Protein	Salt	Dietary Fibre
Mixtures								
Ascophyllum nodosum, Pelvetia canaliculata, and Fucus spiralis[2]	-	-	-	0.21	-	0.10	-	1.67
Ulva spiralis, Palmaria palmata, Saccharina latissima, Porphyra umbilicalis, and *Alaria esculenta*	0.80	0.12	0.25	0.22	0.14	1.31	2.69	5.52
Palmaria palmata, Undaria pinnatifida, Laminaria saccharina, Porphyra spp., and *Ulva lactuca*	0.35	0.13	0.23	0.28	0.03	1.60	1.75	5.98
Brown, Red and Green	0.50	0.02	0.03	1.04	0.02	1.22	5.67	5.73

*Calculated from Dietary Reference Values for Energy (Scientific Advisory Committee on Nutrition, 2015). ** Maximum values taken from a range.

[1] Reported as %RNI for a 2g serving suggestion. [2] Reported as %RNI for a 1g serving suggestion (ingredient) and recommended dose (2x 500mg capsules per day - supplement).

Table 2-5. Nutritional information for a selection of seaweed products sold throughout the UK and the Republic of Ireland.

	%RNI Per 5g Portion																
Seaweed Product	Na	K	Na/K Ratio	Ca	Mg	Mn	Zn	Fe	I	Cu	Vitamin B1	Vitamin B2	Vitamin B3	Vitamin B6	Vitamin B12	Vitamin C	Vitamin E
Brown Seaweed																	
Alaria esculenta **	14.38	11	1.36	8	15	2.33	1.79	7.24	589	2.83	2.75	0.38	0.15	22.14	16667	6.25	-
Ascophyllum nodosum[1]	4.31	1.04	4.14	3.86	5.50	1.67	0.93	2.74	1017	93.33	0.06	0.01	0.69	-	62.67	0.25	0.68
Fucus vesiculosus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Himanthalia elongata	-	-	-	5	14	-	1.32	-	-	-	-	-	-	-	17	-	-
Himanthalia elongata	-	-	-	5	14	-	1.32	-	-	-	-	-	-	-	7	-	-
Laminaria digitata	-	-	-	5	11	-	1.58	-	5	-	-	-	-	-	10	-	-
Laminaria digitata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Laminaria digitata **	16.25	16	1.03	9	13	2.67	1.51	4.02	17857	2.08	2.50	8.46	1.00	30.82	200000	0.23	1.11
Laminaria spp.	-	6	-	7	16	0.68	1.74	2.09	12150	4.50	-	-	-	-	-	-	-
Saccharina latissima	-	-	-	6	12	-	1.58	-	-	-	-	-	-	-	trace	-	-
Saccharina latissima **	10.63	7	1.49	7	12	2.67	1.58	5.75	17857	1.25	-	-	-	-	-	0.23	-
Red Seaweed																	
Chondrus crispus	-	-	-	27	12	-	3.68	12.13	6	-	-	-	-	2.14	7	-	-
Chondrus crispus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chondrus crispus **	8.13	4	1.90	1	14	4.67	3.76	12.07	1071	2.33	-	-	-	-	133333	0.38	-
Palmaria palmata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Palmaria palmata	-	-	-	-	3	-	-	4.83	3	-	-	-	-	0.54	20	-	-
Palmaria palmata	-	13	-		5	19.83	0.68	8.02	468	1.71	-	-	-	-	-	-	-
Palmaria palmata **	9.38	11	0.84	6	8	25.83	1.51	20.11	1964	4.50	3.50	0.96	0.56	3.21	3000000	3.50	-
Porphyra spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Porphyra spp. **	10.00	4	2.26	6	8	13.83	2.16	20.11	1964	3.29	3.00	11.15	2.88	40.00	6666667	13.88	412.50

*Calculated from Dietary Reference Values for Energy (Scientific Advisory Committee on Nutrition, 2015). ** Maximum values taken from a range.

[1] Reported as %RNI for a 2g serving suggestion. [2] Reported as %RNI for a 1g serving suggestion (ingredient) and recommended dose (2x 500mg capsules per day - supplement).

Table 2-5 continued. Nutritional information for a selection of seaweed products sold throughout the UK and the Republic of Ireland.

	%RNI Per 5g Portion															
Seaweed Product	Na	K	Na/K Ratio	Ca	Mg	Mn	Zn	Fe	I	Cu	Vitamin B1	Vitamin B2	Vitamin B3	Vitamin B6	Vitamin B12	Vitamin C	Vitamin E
Mixtures																	
Ascophyllum nodosum, Pelvetia canaliculata, and Fucus spiralis[2]	2.18	0.55	3.94	1.76	2.68	1.50	0.75	1.60	279	62.50	0.06	0.01	0.61	-	0.09	0.17	1.55
Palmaria palmata, Undaria pinnatifida, Laminaria saccharina, Porphyra spp., and *Ulva lactuca*	-	-	-	8.54	5.65	-	1.32	-	1.46	-	-	-	-	1.79	3	-	2.25
Brown, Red and Green	-	8.81	-	43.61	12.75	8.33	2.27	9.33	1464	1.67	-	-	-	-	-	-	-

*Calculated from Dietary Reference Values for Energy (Scientific Advisory Committee on Nutrition, 2015). ** Maximum values taken from a range.

[1] Reported as %RNI for a 2g serving suggestion. [2] Reported as %RNI for a 1g serving suggestion (ingredient) and recommended dose (2x 500mg capsules per day - supplement).

Table 2-6. Amino acid content of brown seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Alaria esculenta [1]	2.00	4.60	7.70	6.10	1.30	5.30	6.40	-	6.80	13.4	5.40	-	6.40	4.60	4.00	13.6	16.0	5.80	Munda and Gubenšek (1976)
Alaria esculenta [1]	1.60	3.80	7.50	5.30	2.40	4.80	5.10	-	5.50	18.9	4.80	-	5.70	5.10	2.90	8.40	20.1	5.20	Maehre et al. (2014)
Ascophyllum nodosum	1.06	3.12	5.65	4.17	1.96	3.67	4.02	-	4.12	5.20	3.41	-	4.27	3.06	1.92	8.41	13.1	3.77	Munda (1977)
Chnoospora minima	2.20	4.20	8.10	5.30	2.20	5.10	5.40	-	5.90	8.10	4.20	-	6.20	4.50	2.10	12.2	14.8	6.20	Lourenço et al. (2002)
Chorda filum [1]	0.80	1.70	3.10	2.20	1.60	2.00	2.50	-	2.00	3.60	2.30	-	3.00	2.20	1.40	5.20	5.30	2.40	Munda and Gubenšek (1976)
Chordaria flagelliformis [1]	1.90	4.50	7.10	5.10	2.70	4.80	5.00	-	6.00	8.80	5.20	-	6.20	4.80	2.90	9.30	11.2	4.40	Munda and Gubenšek (1976)
Colpomenia sinuosa	1.58	1.09	3.34	0.74	2.95	0.97	1.05	-	2.46	3.32	-	-	2.09	3.32	2.29	3.85	6.06	1.92	Tabarsa et al. (2012)
Desmarestia aculeata [1]	0.80	1.80	2.90	1.80	1.80	1.80	2.50	-	2.30	2.90	1.90	-	2.60	1.60	1.40	4.90	4.90	2.40	Munda and Gubenšek (1976)
Dictyosiphon foeniculaceus [1]	1.70	3.70	5.80	4.70	2.20	3.80	3.80	-	4.60	5.90	4.70	-	4.90	3.80	2.40	9.00	9.10	3.60	Munda and Gubenšek (1976)
Dictyota dichotoma	1.67	2.45	4.80	3.34	0.41	2.75	2.25	-	3.29	3.16	-	-	3.36	2.76	1.86	5.81	8.47	2.60	Tabarsa et al. (2012)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen.

Table 2-6 continued. Amino acid content of brown seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Dictyota menstrualis	2.20	4.70	8.70	5.40	1.30	5.40	5.30	-	5.70	6.90	5.40	-	6.10	4.80	2.30	13.8	12.7	6.10	Lourenço et al. (2002)
Durvillaea antarctica (leaves)	0.75	0.35	0.60	0.51	0.91	0.37	0.26	-	0.46	0.45	0.33	<0.01	0.22	<0.01	0.18	0.75	1.05	0.43	Ortiz et al. (2006)
Durvillaea antarctica (stem)	1.18	0.16	0.27	0.19	0.42	0.19	0.28	-	0.19	0.83	0.15	0.10	0.29	<0.01	0.08	2.02	0.97	0.26	Ortiz et al. (2006)
Ectocarpus siliculosus [1]	1.40	4.10	6.80	3.90	2.50	4.50	4.90	-	5.00	7.70	4.40	-	5.60	3.90	3.10	9.80	10.7	4.70	Munda and Gubenšek (1976)
Fucus ceranoides	1.24	2.77	4.80	3.54	0.74	2.99	3.82	-	3.54	6.08	3.94	-	3.63	2.76	2.01	9.88	31.5	3.58	Munda (1977)
Fucus vesiculosus	1.37	3.21	6.05	4.08	1.81	3.89	3.65	-	4.26	5.81	4.38	-	4.50	3.47	1.85	8.55	14.7	3.65	Munda (1977)
Fucus vesiculosus [1]	1.10	2.70	5.00	4.30	1.50	3.30	3.40	-	3.70	5.00	3.20	-	3.80	3.10	1.50	8.30	17.9	3.50	Maehre et al. (2014)
Fucus vesiculosus [1]	0.50	0.70	1.20	0.90	-	0.80	1.20	-	0.70	1.20	0.70	-	1.40	0.50	0.50	2.70	2.40	1.20	Munda and Gubenšek (1976)
Himanthalia elongata [1]	1.00	2.10	3.80	2.90	1.30	2.80	2.60	-	3.10	3.40	2.60	0.31	2.70	1.80	2.70	5.20	6.80	2.90	Cofrades et al. (2010)
Hizikia fusiforme [2]	2.60	4.00	6.70	3.10	1.60	4.60	4.10	0.40	4.90	4.30	4.50	0.90	4.80	3.80	2.80	9.10	18.7	3.70	Dawczynski et al. (2007)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen.

Table 2-6 continued. Amino acid content of brown seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Laminaria digitata [1]	1.20	2.70	5.20	3.70	1.80	3.40	3.80	-	3.60	5.20	3.40	-	4.10	3.90	1.80	6.20	8.50	3.60	Munda (1977)
Laminaria digitata [1]	1.20	3.10	4.60	3.20	1.30	3.10	3.30	-	4.10	3.60	2.80	-	3.80	2.80	2.40	7.60	7.40	2.70	Munda and Gubenšek (1976)
Laminaria hyperborea [1]	1.20	2.20	4.50	3.40	1.60	3.10	3.50	-	3.50	6.20	3.00	-	3.80	3.50	1.60	5.90	8.60	3.50	Maehre et al. (2014)
Laminaria sp. [2]	2.20	2.70	4.90	3.90	0.90	3.20	3.50	0.50	3.80	5.70	3.30	1.20	4.00	3.10	1.70	12.5	23.8	3.30	Dawczynski et al. (2007)
Padina gymnospora	2.50	4.70	8.80	5.70	1.00	5.60	5.40	-	5.70	7.20	5.30	-	6.30	4.60	2.50	13.1	13.4	5.40	Lourenço et al. (2002)
Padina pavonica	3.12	4.32	8.62	4.55	1.05	4.33	5.66	-	6.96	7.19	-	-	7.37	5.44	4.39	12.7	17.1	5.23	Tabarsa et al. (2012)
Pelvetia canaliculata [1]	0.50	0.90	1.70	1.30	1.00	1.80	1.80	-	2.20	2.20	1.00	-	2.30	1.50	0.70	4.10	10.7	2.00	Munda and Gubenšek (1976)
Pelvetica canaliculata [1]	1.00	3.00	5.20	3.70	1.40	3.40	3.50	-	3.90	5.50	3.20	-	4.10	3.20	1.40	5.90	15.0	3.60	Maehre et al. (2014)
Sargassum naozhouense	1.07	4.02	6.52	3.66	2.41	4.38	3.93	0.89	4.64	5.27	4.20	0.54	4.38	3.30	2.95	8.39	13.2	3.21	Peng et al. (2013)
Sargassum polycystum	0.26	2.94	4.67	2.11	1.25	30.4	2.60	-	3.13	4.25	2.88	-	3.19	2.55	1.26	4.47	8.08	2.58	Matanjun et al. (2009)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen.

Table 2-6 continued. Amino acid content of brown seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Sargassum vulgare	2.10	4.80	8.50	5.40	2.20	5.30	4.80	-	5.80	7.20	4.30	-	5.70	4.60	2.20	10.9	17.6	5.10	Lourenço et al. (2002)
Scytosiphon lomentaria [1]	1.60	4.20	7.20	4.70	2.40	4.30	5.40	-	4.80	7.10	4.90	-	6.80	4.40	3.10	10.0	11.9	5.00	Munda and Gubenšek (1976)
Undaria pinnatifia [2]	2.50	4.10	7.40	5.60	1.70	4.70	4.40	0.70	5.20	4.70	5.20	0.90	5.10	3.60	2.90	8.70	14.5	4.00	Dawczynski et al. (2007)
Undaria pinnatifida (Blade) [1]	6.60	4.23	7.50	6.43	3.08	4.05	3.85	-	5.85	6.35	5.10	-	6.43	-	6.33	10.8	13.2	5.40	Zhou et al. (2015)
Undaria pinnatifida (Sporophyll) [1]	6.33	4.33	7.18	5.28	3.13	5.13	3.78	-	6.53	11.9	4.20	-	7.70	-	5.63	9.58	12.9	5.48	Zhou et al. (2015)
Undaria pinnatifida [1]	6.80	3.50	5.30	2.40	3.50	5.50	6.00	-	7.30	8.60	6.50	0.82	6.40	4.80	10.0	12.2	13.5	6.20	Cofrades et al. (2010)
Undaria pinnatifida [1]	1.71	5.08	8.61	4.00	0.14	4.85	2.92	-	5.85	9.76	8.82	0.33	6.58	4.43	2.10	7.56	12.1	4.18	Taboada et al. (2013)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen.

Table 2-7. Amino acid content of red seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Acanthophora spicifera	1.70	4.10	7.40	7.20	0.70	4.80	5.50	-	5.40	6.00	5.10	-	5.00	4.10	2.70	14.4	16.9	4.80	Lourenço et al. (2002)
Aglaothamnion uruguayense	2.00	4.80	8.00	6.60	0.60	5.10	5.70	-	6.10	7.60	4.80	-	6.60	5.10	2.60	12.6	15.8	5.40	Lourenço et al. (2002)
Ahnfeltia plicata [3]	1.00	3.50	5.40	6.60	1.40	3.70	4.40	-	5.80	5.50	15.4	3.20	6.50	6.00	4.60	10.5	9.40	5.10	Parjikolaei et al. (2016)
Callithamnion arbuscula [1]	3.70	13.9	21.6	14.1	9.40	14.0	16.3	-	17.1	20.6	13.2	1.60	17.3	13.7	11.1	32.1	38.1	15.6	Munda and Gubenšek (1976)
Chondrus crispus [3]	2.10	3.90	6.60	6.30	1.80	5.20	4.80	-	5.40	6.70	9.00	3.40	6.70	5.80	2.40	11.2	11.7	5.70	Parjikolaei et al. (2016)
Corallina officinalis [1]	0.50	1.40	2.10	1.90	0.90	1.50	1.50	-	2.00	2.10	1.70	0.40	2.40	1.20	1.10	4.70	3.70	1.80	Munda and Gubenšek (1976)
Cryptonemia seminervis	2.20	4.60	7.90	8.10	1.00	6.10	5.70	-	6.20	6.50	8.10	-	5.40	6.40	2.70	10.1	13.0	4.60	Lourenço et al. (2002)
Cystoclonium purpureum [1]	3.40	10.5	15.2	12.3	5.80	9.90	10.6	-	13.1	13.5	12.9	5.10	12.7	8.90	7.80	21.8	23.4	11.3	Munda and Gubenšek (1976)
Delesseria sanguinea [3]	1.50	3.90	6.00	5.50	1.70	4.10	4.90	-	5.00	6.30	6.60	2.40	6.10	12.7	2.90	10.6	14.4	5.20	Parjikolaei et al. (2016)
Dilsea carnosa [1]	2.00	6.30	9.00	7.00	3.10	6.10	6.80	-	7.80	8.30	7.70	0.70	7.70	5.50	4.00	13.6	17.1	6.70	Munda and Gubenšek (1976)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen. [3] Expressed as % total amino acid.

Table 2-7 continued. Amino acid content of red seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Dilsea carnosa [3]	1.60	3.60	6.40	5.60	1.60	4.50	4.70	-	4.80	7.70	13.5	2.60	4.70	5.30	0.60	15.4	10.8	5.90	Parjikolaei et al. (2016)
Eucheuma cottonii	0.25	2.41	3.37	1.45	0.83	19.1	2.09	-	2.61	3.14	2.60	-	2.27	2.02	1.01	2.65	5.17	1.92	Matanjun et al. (2009)
Furcellaria lumbricalis [3]	1.40	3.80	6.10	4.90	1.40	5.90	4.70	-	5.70	5.70	16.2	3.70	6.10	6.50	0.70	9.30	10.7	5.60	Parjikolaei et al. (2016)
Gelidium microdon	-	-	-	5.32	4.17	4.42	-	-	-	-	8.48	-	-	-	-	-	-	-	Paiva et al. (2016)
Gigartina stellata [1]	2.50	4.60	7.20	9.10	2.50	5.00	5.60	-	5.60	7.00	9.00	1.20	9.80	4.60	6.50	13.2	12.6	6.30	Munda and Gubenšek (1976)
Gracilaria domingensis	2.90	4.10	8.80	5.70	0.70	5.70	6.10	-	5.60	8.10	4.70	-	6.60	5.10	2.30	12.2	12.6	5.30	Lourenço et al. (2002)
Gracilaria edulis	3.30	1.10	0.37	1.91	1.69	1.31	2.98	-	1.71	2.64	3.27	-	0.73	-	0.56	4.64	13.1	0.84	Sakthivel and Devi (2015)
Gracilaria salicornia	1.43	3.03	7.66	7.71	7.75	3.27	3.29	-	4.14	7.55	7.58	-	7.56	3.98	7.59	5.39	7.59	3.46	Tabarsa et al. (2012)
Gracilaria tenuifrons	2.40	4.80	8.20	6.60	1.30	5.10	5.60	-	6.10	7.40	6.00	-	6.30	4.20	2.40	11.5	13.8	5.20	Lourenço et al. (2002)
Gracilaria vermiculophylla [3]	1.50	4.30	7.60	5.60	2.10	4.90	5.60	-	5.60	6.90	8.30	2.40	6.20	5.40	2.40	11.4	12.4	6.20	Parjikolaei et al. (2016)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen. [3] Expressed as % total amino acid.

Table 2-7 continued. Amino acid content of red seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)										
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Hypnea charoides	0.66	4.85	7.23	6.49	1.68	5.60	5.13	-	6.14	5.23	6.36	2.81	5.06	4.79	2.60	8.86	9.84	4.49	Wong and Cheung (2000)
Hypnea japonica	0.69	4.48	9.79	6.66	1.85	3.72	4.59	-	5.63	5.74	6.68	2.91	5.42	4.54	2.79	9.84	11.0	4.75	Wong and Cheung (2000)
Hypnea musciformis	0.58	4.82	7.74	5.53	1.46	3.08	4.15	-	5.37	5.24	5.19	-	4.92	4.08	2.58	7.63	11.9	3.23	Siddique (2013)
Hypnea pannosa	0.64	4.21	8.48	4.56	1.62	3.29	6.27	-	5.58	4.79	6.83	-	4.77	4.42	2.62	8.65	10.7	4.39	Siddique (2013)
Laurencia flagellifera	1.50	4.60	7.70	10.2	0.50	4.70	5.40	-	6.00	6.80	4.30	-	5.60	4.20	3.70	13.0	15.3	5.10	Lourenço et al. (2002)
Ochtodes secundiramea [1]	0.16	0.36	0.77	0.60	0.18	0.47	0.46	0.27	0.41	0.76	0.59	0.10	0.54	0.47	0.30	1.13	1.08	0.49	Tabarsa et al. (2012)
Odonthalia dentata [3]	1.60	4.40	6.40	6.90	1.70	5.10	5.20	-	5.60	5.90	6.10	3.00	5.70	13.6	1.30	11.4	10.4	5.70	Parjikolaei et al. (2016)
Palmaria palmata [1]	1.80	6.50	11.3	8.90	3.10	7.10	7.10	-	9.60	12.2	6.00	0.50	9.60	9.70	4.70	13.1	21.3	8.40	Maehre et al. (2014)
Palmaria palmata [1]	2.60	6.70	10.2	8.30	3.90	7.10	8.70	-	10.5	13.2	8.70	-	10.2	9.70	5.60	18.4	10.0	7.60	Munda and Gubenšek (1976)
Palmaria palmata [3]	1.80	3.80	6.50	7.00	2.10	4.50	4.40	-	6.10	7.50	6.10	3.50	6.30	4.70	0.90	13.3	16.0	5.50	Parjikolaei et al. (2016)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen. [3] Expressed as % total amino acid.

Table 2-7 continued. Amino acid content of red seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Phycodrys rubens [3]	1.70	4.20	6.60	6.20	1.80	4.60	5.00	-	5.80	7.80	6.90	2.50	7.20	8.20	2.50	11.8	11.4	5.60	Parjikolaei et al. (2016)
Plocamium brasiliense	2.10	5.40	8.10	7.90	0.40	6.80	5.60	-	6.70	7.90	6.70	-	6.80	5.20	2.30	12.4	11.2	6.00	Lourenço et al. (2002)
Plocamium Brasiliense	0.21	0.53	1.01	0.70	0.25	0.68	0.64	0.27	0.59	1.22	0.76	0.17	0.81	0.59	0.46	1.58	1.54	0.74	Tabarsa et al. (2012)
Plumaria elegans [1]	3.40	10.1	16.1	12.6	6.00	10.7	13.8	-	11.5	16.1	16.0	4.20	19.7	13.7	9.20	39.9	29.7	14.1	Munda and Gubenšek (1976)
Polysiphonia lanosa [1]	1.70	7.00	11.0	12.1	5.70	17.6	9.90	-	7.50	9.10	10.4	2.30	10.4	6.80	8.70	20.2	19.6	10.2	Munda and Gubenšek (1976)
Porphyra acanthophora	3.20	4.40	8.60	6.70	1.20	5.00	6.20	-	6.80	9.40	5.10	-	7.50	4.90	2.50	13.3	13.7	5.70	Lourenço et al. (2002)
Porphyra columbina	1.26	2.71	7.38	6.01	1.68	3.70	5.91	0.63	5.85	12.5	6.19	1.89	8.87	3.96	2.55	12.2	10.5	6.16	Cian et al. (2014)
Porphyra linearis [1]	5.70	12.3	23.8	20.6	9.50	11.7	19.2	-	18.8	40.6	24.9	-	18.5	12.2	10.0	34.4	39.5	18.1	Munda and Gubenšek (1976)
Porphyra purpurea [1]	2.20	3.44	5.32	2.99	1.37	7.82	5.01	-	4.80	8.05	9.00	0.46	7.54	3.80	2.94	6.66	8.30	4.63	Taboada et al. (2013)
Porphyra purpurea [1]	4.00	10.0	17.9	13.4	5.70	9.60	13.7	-	19.7	30.3	16.0	-	14.9	12.3	10.0	24.9	29.7	12.6	Munda and Gubenšek (1976)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen. [3] Expressed as % total amino acid.

Table 2-7 continued. Amino acid content of red seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)									
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Porphyra sp. [2]	2.60	3.10	5.50	4.90	1.80	3.30	5.30	0.70	5.20	6.20	5.90	1.20	5.10	3.50	3.40	8.50	10.2	4.00	Dawczynski et al. (2007)
Porphyra sp. [2]	2.40	3.30	5.90	5.20	1.70	3.50	5.20	0.70	4.50	4.20	5.90	1.30	4.10	3.60	3.20	8.20	9.30	4.90	Dawczynski et al. (2007)
Porphyra umbilicalis [1]	5.70	13.3	28.0	20.5	4.50	16.8	21.1	-	24.5	39.5	24.0	3.20	23.9	16.9	17.1	37.8	44.1	23.6	Cofrades et al. (2010)
Pterocladiella capillacea	4.40	3.30	6.10	9.30	1.10	5.10	5.00	-	4.70	5.60	5.10	-	5.30	4.90	3.80	10.7	15.6	5.30	Lourenço et al. (2002)
Pterocladiella capillacea	-	-	-	5.34	4.21	5.24	-	-	-	-	5.46	-	-	-	-	-	-	-	Paiva et al. (2016)
Vertebrata lanosa [1]	2.00	7.20	9.90	12.6	1.80	8.20	7.80	-	7.60	7.60	7.00	2.10	8.90	10.8	5.40	12.3	16.3	7.70	Maehre et al. (2014)

[1] Expressed as g/100g DW. [2] Expressed as g/16g Nitrogen. [3] Expressed as % total amino acid.

Table 2-8. Amino acid content of green seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)										
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Acrosiphonia sp. [1]	1.60	6.90	9.60	8.00	5.10	7.40	8.10	-	10.6	13.2	8.90	-	12.6	7.80	5.60	19.3	17.6	8.30	Munda and Gubenšek (1976)
Caulerpa fastigiata	2.20	4.00	8.70	7.10	1.50	6.60	4.80	-	6.10	6.20	5.50	-	7.10	7.70	3.90	10.1	10.7	6.20	Munda and Gubenšek (1976)
Caulerpa lentillifera	1.44	5.06	7.79	1.22	1.58	20.0	5.84	-	6.18	6.88	5.71	-	5.14	4.29	3.33	8.33	13.5	5.49	Matanjun et al. (2009)
Caulerpa lentillifera	0.80	6.20	9.90	8.20	-	6.10	7.90	-	8.70	8.50	8.70	-	8.50	5.70	4.80	14.3	17.8	7.60	Ratana-arporn and Chirapart (2006)
Caulerpa racemosa	2.90	4.10	8.30	6.50	1.00	5.40	5.70	-	5.70	6.50	5.10	-	6.80	4.60	2.60	9.90	14.6	5.40	Lourenço et al. (2002)
Cladophora rupestris [1]	0.70	1.60	2.70	2.10	0.90	2.10	2.20	-	2.40	3.10	2.50	0.50	3.30	2.90	1.50	3.50	5.70	2.20	Maehre et al. (2014)
Cladophora rupestris [1]	4.30	11.0	16.0	14.4	7.30	11.2	12.5	-	16.0	15.5	14.9	6.70	18.2	14.1	11.6	40.6	34.4	11.9	Munda and Gubenšek (1976)
Codium decorticatum	3.50	4.00	8.50	6.40	0.70	5.10	6.10	-	6.30	8.90	5.20	-	7.30	4.90	2.30	10.8	12.0	5.20	Lourenço et al. (2002)
Codium spongiosum	2.30	4.40	8.40	6.80	0.80	5.40	5.40	-	6.60	8.10	4.00	-	6.10	4.60	2.30	12.0	14.1	5.30	Lourenço et al. (2002)
Codium taylorii	2.70	4.40	8.20	7.50	2.00	6.10	4.50	-	6.90	6.70	3.80	-	5.40	7.90	2.80	10.6	11.3	5.80	Lourenço et al. (2002)

[1] Expressed as g/100g DW.

Table 2-8 continued. Amino acid content of green seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)										
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Enteromorpha ahlneriana [1]	0.90	4.10	5.60	3.50	2.40	4.30	5.40	-	6.10	8.30	4.50	-	5.90	3.70	2.40	9.80	10.6	4.50	Munda and Gubenšek (1976)
Enteromorpha sp.	2.24	3.22	4.43	3.20	2.64	3.71	4.04	-	5.50	4.81	3.45	4.25	3.49	4.72	2.69	4.66	8.58	5.25	Aguilera-Morales et al. (2005)
Ulva compressa	-	-	-	11.3	6.55	8.45	-	-	-	-	14.1	-	-	-	-	-	-	-	Paiva et al. (2016)
Ulva fasciata	2.40	3.90	7.60	5.10	0.90	5.10	5.10	-	5.70	8.50	5.60	-	6.50	4.60	3.30	13.0	12.6	5.80	Lourenço et al. (2002)
Ulva intestinalis [1]	2.10	5.90	9.50	6.40	2.30	7.40	8.00	-	8.40	14.7	7.40	1.40	8.50	6.60	3.80	14.6	18.2	7.80	Maehre et al. (2014)
Ulva lactuca	0.48	3.82	6.71	6.58	1.57	3.50	5.06	-	5.50	7.39	8.44	1.33	6.74	4.46	3.50	9.87	8.73	5.54	Wong and Cheung (2000)
Ulva lactuca	1.00	4.00	7.40	4.60	2.20	5.40	4.70	0.70	5.80	7.80	7.60	1.10	5.90	5.50	3.60	13.8	13.2	4.90	Bikker et al. (2016)
Ulva lactuca	1.39	4.76	8.25	6.50	2.38	2.70	6.31	-	9.15	9.16	6.19	1.94	6.65	4.12	5.99	12.9	12.9	6.94	Yaich et al. (2011)
Ulva lactuca [1]	1.60	4.40	85.0	5.10	2.20	6.00	6.20	-	7.10	10.1	6.00	1.00	7.30	5.80	3.40	9.00	12.2	5.90	Maehre et al. (2014)
Ulva lactuca [1]	3.70	13.9	21.6	14.1	9.40	14.4	16.3	-	17.1	20.6	17.2	1.60	17.3	13.7	11.1	32.1	38.1	15.6	Munda and Gubenšek (1976)

[1] Expressed as g/100g DW.

Table 2-8 continued. Amino acid content of green seaweeds.

	Essential Amino Acids (g/100g protein)								Non-Essential Amino Acids (g/100g protein)										
Seaweed	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Ala	Arg	Cys	Gly	Pro	Tyr	Asn + Asp	Gln + Glu	Ser	Author
Ulva lactuca	1.34	5.50	10.4	7.23	6.72	12.5	7.98	-	3.39	11.0	4.87	0.55	8.16	< 0.01	4.35	14.9	15.1	8.33	Ortiz et al. (2006)
Ulva lactuca	1.52	2.17	4.51	2.54	0.59	2.84	3.11	-	3.92	4.33	3.79	-	3.97	3.79	2.34	4.97	7.07	2.87	Tabarsa et al. (2012)
Ulva reticulata	2.30	9.00	16.8	12.8	-	11.2	11.5	-	13.4	17.2	18.4	-	13.8	10.8	7.70	26.6	27.6	13.6	Ratana-arporn and Chirapart (2006)
Ulva rigida	1.62	3.72	6.63	3.49	2.03	4.78	3.17	-	4.54	6.05	9.09	1.00	4.98	2.43	3.78	7.63	9.79	3.40	Taboada et al. (2013)
Ulva rigida	-	-	-	2.38	1.43	1.78	-	-	-	-	2.99	-	-	-	-	-	-	-	Paiva et al. (2016)

Table 2-9. Lipid content of brown seaweeds.

Seaweed	Country	Harvested	Total Lipid (g/kg DW)	5g portion (%RNI)	Saturates (% FA)	MUFAs (% FA)	PUFAs (% FA)	PUFA/SFA Ratio	n-3 PUFAs (% FA)	n-6 PUFAs (% FA)	n-6/n-3 Ratio	Trans Fats (% FA)	Author
Alaria esculenta	Norway	June	15.0	0.11	37.4	25.4	33.2	0.89	20.4	12.8	0.63	-	Maehre et al. (2014)
Ascophyllum nodosum	Norway	September/ October	12.6*	0.09	-	-	30.0	-	8.0	22.0	2.75	-	van Ginneken et al. (2011)
Colpomenia sinuosa	Iran	-	15.0	0.11	51.9	32.9	15.3	0.29	7.7	7.6	0.99	-	Rohani-Ghadikolaei et al. (2012)
Durvillaea antarctica	Chile	November	8.0	0.06	25.8	38.1	34.4	1.33	10.8	22.0	2.04	0.3	Ortiz et al. (2006)
Fucus vesiculosis	Norway	June	26.5	0.19	24.3	47.1	25.8	1.06	8.6	17.2	2.00	-	Maehre et al. (2014)
Fucus serratus	France	September/ October	37.4*	0.27	-	-	31.0	-	9.0	22.0	2.44	-	van Ginneken et al. (2011)
Fucus spiralis	Portugal	January	52.3	0.37	27.1	39.0	15.7	0.58	14.0	25.0	1.78	6.4	Paiva et al. (2014)
Himanthalia elongata	Spain	-	-	-	39.0	17.9	43.0	1.10	34.7	8.1	0.23	-	Cofrades et al. (2010)
Himanthalia elongata	Spain	July	9.7	0.07	39.1	22.8	38.2	0.98	18.7	15.1	0.81	-	Sanchez-Machado et al. (2004)
Laminaria digitata	Norway	June	8.5	0.06	31.5	18.7	45.6	1.45	29.1	16.5	0.57	-	Maehre et al. (2014)
Laminaria hyperborea	France	September/ October	18.1*	0.13	-	-	53.0	-	39.0	14.0	0.36	-	van Ginneken et al. (2011)
Laminaria hyperborea	Norway	June	11.4	0.08	33.7	26.5	34.2	1.01	21.5	12.7	0.59	-	Maehre et al. (2014)
Laminaria ochroleuca	Spain	July	9.2	0.07	33.8	19.2	46.9	1.39	25.1	21.0	0.84	-	Sanchez-Machado et al. (2004)
Pelvetia canaliculata	Norway	June	37.4	0.27	21.2	42.2	34.0	1.60	9.3	24.6	2.65	-	Maehre et al. (2014)

* Total Fatty Acids (g/kg DW)

Table 2-9 continued. Lipid content of brown seaweeds.

Seaweed	Country	Harvested	Total Lipid (g/kg DW)	5g portion (%RNI)	Saturates (% FA)	MUFAs (% FA)	PUFAs (% FA)	PUFA/SFA Ratio	n-3 PUFAs (% FA)	n-6 PUFAs (% FA)	n-6/n-3 Ratio	Trans Fats (% FA)	Author
Sargassum ilicifolium	Iran	-	20.0	0.14	55.2	27.5	17.4	0.32	8.2	9.2	1.12	-	Rohani-Ghadikolaei et al. (2012)
Sargassum naozhouense	China	July	10.6	0.08	33.6	10.4	18.8	0.56	1.6	13.2	8.25	4.0	Peng et al. (2013)
Sargassum natans	North Atlantic Ocean	September/ October	44.7*	0.32	-	-	31.0	-	20.0	11.0	0.55	-	van Ginneken et al. (2011)
Sargassum polycystum	Malaysia, Brunei, Indonesia	-	2.9	0.02	51.3	28.4	20.3	0.40	9.6	9.4	0.98	-	Matanjun et al. (2009)
Undaria pinnatifida	Ireland	September/ October	14.5*	0.10	-	-	56.0	-	35.0	21.0	0.60	-	van Ginneken et al. (2011)
Undaria pinnatifida	Spain	-	-	-	39.0	14.7	46.3	1.19	36.5	9.8	0.27	-	Cofrades et al. (2010)
Undaria pinnatifida	Spain	April	10.5	0.08	20.4	10.5	69.1	3.39	44.7	22.1	0.49	-	Sanchez-Machado et al. (2004)

* Total Fatty Acids (g/kg DW)

Table 2-10. Lipid content of red seaweeds.

Seaweed	Country	Harvested	Total Lipid (g/kg DW)	5g portion (%RNI)	Saturates (% FA)	MUFAs (% FA)	PUFAs (% FA)	PUFA/SFA Ratio	n-3 PUFAs (% FA)	n-6 PUFAs (% FA)	n-6/n-3 Ratio	Trans Fats (% FA)	Author
Chondrus crispus	France	September/ October	14.7*	0.10	-	-	42.0	-	22.0	20.0	0.91	-	van Ginneken et al. (2011)
Eucheuma cottinii	Malaysia, Brunei, Indonesia	-	11.0	0.08	25.2	23.3	51.6	2.05	45.7	4.7	0.10	-	Matanjun et al. (2009)
Gracilaria corticata	Iran	-	18.0	0.13	58.7	22.0	19.1	0.33	7.8	11.2	1.44	-	Rohani-Ghadikolaei et al. (2012)
Gracilaria salicornia	Iran	April	20.0	0.14	48.9	16.4	17.3	0.35	8.0	10.1	1.26	-	Tabarsa et al. (2012)
Gracilaria gracilis	Italy	January	4.2*	0.03	34.9	12.5	51.8	1.48	2.4	49.8	20.75	-	Francavilla et al. (2013)
Gracilaria gracilis	Italy	October	3.1*	0.02	51.7	25.3	22.5	0.44	3.7	18.9	5.11	-	Francavilla et al. (2013)
Gracilaria gracilis	Italy	July	4.7*	0.03	37.3	20.0	42.7	1.14	4.3	38.1	8.86	-	Francavilla et al. (2013)

* Total Fatty Acids (g/kg DW)

Table 2-10 continued. Lipid content of red seaweeds.

Seaweed	Country	Harvested	Total Lipid (g/kg DW)	5g portion (%RNI)	Saturates (% FA)	MUFAs (% FA)	PUFAs (% FA)	PUFA/SFA Ratio	n-3 PUFAs (% FA)	n-6 PUFAs (% FA)	n-6/n-3 Ratio	Trans Fats (% FA)	Author
Gracilaria gracilis	Italy	April	6.7*	0.05	34.2	18.6	47.2	1.38	9.5	37.5	3.95	-	Francavilla et al. (2013)
Hypnea valentiae	Iran	-	28.0	0.20	67.4	23.6	9.2	0.14	2.5	6.7	2.68	-	Rohani-Ghadikolaei et al. (2012)
Ochtodes secundirame a	Brasil	October	35.4 (12.1*)	0.25	66.1	3.3	15.7	0.24	6.6	9.1	1.38	0.8	Gressler et al. (2011)
Osmundea pinnatifida	Portugal	January	75.3	0.54	20.6	20.6	22.2	1.08	17.0	5.2	0.31	1.0	Paiva et al. (2014)
Palmaria palmata	Ireland	September/ October	18.2*	0.14	-	-	66.0	-	63.0	3.0	0.05	-	van Ginneken et al. (2011)
Palmaria palmata	Norway	June	13.9	0.10	43.7	8.7	35.4	0.81	34.3	1.1	0.03	-	Maehre et al. (2014)
Palmaria spp.	Spain	September	18.0	0.13	60.5	10.7	28.9	0.48	25.5	2.1	0.08	-	Sanchez-Machado et al. (2004)

* Total Fatty Acids (g/kg DW)

Table 2-10 continued. Lipid content of red seaweeds.

Seaweed	Country	Harvested	Total Lipid (g/kg DW)	5g portion (%RNI)	Saturates (% FA)	MUFAs (% FA)	PUFAs (% FA)	PUFA/SFA Ratio	n-3 PUFAs (% FA)	n-6 PUFAs (% FA)	n-6/n-3 Ratio	Trans Fats (% FA)	Author
Plocamium brasiliense	Brasil	October	37.4 (9.3*)	0.27	74.2	4.3	20.4	0.27	8.6	11.8	1.37	1.1	Gressler et al. (2011)
Porphyra umbilicalis	Spain	-	-	-	35.7	9.5	56.4	1.58	50.7	5.6	0.11	-	Cofrades et al. (2010)
Porphyra spp.	Spain	August	10.3	0.07	65.0	18.9	16.1	0.25	7.2	8.0	1.11	-	Sanchez-Machado et al. (2004)
Porphyra spp.	Portugal	January	88.8	0.63	17.3	25.0	18.4	1.06	9.8	15.2	1.55	4.2	Paiva et al. (2014)
Vertebrata lanosa	Norway	June	18.0	0.13	28.6	22.6	45.8	1.60	33.8	11.9	0.35	-	Maehre et al. (2014)

* Total Fatty Acids (g/kg DW)

Table 2-11. Lipid content of green seaweeds.

Seaweed	Country	Harvested	Total Lipid (g/kg DW)	5g portion (%RNI)	Saturates (% FA)	MUFAs (% FA)	PUFAs (% FA)	PUFA/SFA Ratio	n-3 PUFAs (% FA)	n-6 PUFAs (% FA)	n-6/n-3 Ratio	Trans Fats (% FA)	Author
Caulerpa lentillifera	Malaysia, Brunei, Indonesia	-	11.1	0.08	46.4	36.8	16.8	0.36	7.6	8.0	1.05	-	Matanjun et al. (2009)
Caulerpa taxifolia	Indonesia	September/October	7.3*	0.05	-	-	42.0	-	38.0	4.0	0.11	-	van Ginneken et al. (2011)
Cladophora rupestris	Norway	June	8.8	0.06	40.8	27.3	21.5	0.53	14.7	7.4	0.50	-	Maehre et al. (2014)
Enteromorpha spp.	Mexico	Winter 1997	22.4	0.16	-	-	21.3	-	10.4	10.9	1.05	-	Aguilera-Morales et al. (2005)
Enteromorpha spp.	Mexico	Winter 1998	22.7	0.16	-	-	18.4	-	10.2	8.3	0.81	-	Aguilera-Morales et al. (2005)
Ulva intestinalis	Iran	-	29.0	0.21	60.6	24.8	14.8	0.24	9.8	4.8	0.49	-	Rohani-Ghadikolaei et al. (2012)
Ulva intestinalis	Norway	June	22.0	0.16	25.0	22.4	37.1	1.48	31.3	5.8	0.19	-	Maehre et al. (2014)
Ulva lactuca	Iran	-	36.0	0.26	66.3	23.8	9.8	0.15	4.8	5.1	1.06	-	Rohani-Ghadikolaei et al. (2012)

* Total Fatty Acids (g/kg DW)

Table 2-11 continued. Lipid content of green seaweeds.

Seaweed	Country	Harvested	Total Lipid (g/kg DW)	5g portion (%RNI)	Saturates (% FA)	MUFAs (% FA)	PUFAs (% FA)	PUFA/SFA Ratio	n-3 PUFAs (% FA)	n-6 PUFAs (% FA)	n-6/n-3 Ratio	Trans Fats (% FA)	Author
Ulva lactuca	Chile	November	3.0	0.02	33.8	36.7	18.2	0.54	6.6	8.7	1.32	0.4	Ortiz et al. (2006)
Ulva lactuca	Iran	April	9.9	0.07	48.3	5.1	24.8	0.51	11.4	13.4	1.18	-	Tabarsa et al. (2012)
Ulva lactuca	Tunisia	July	78.7	0.56	69.0	24.3	6.7	0.10	-	-	-	-	Yaich et al. (2011)
Ulva lactuca	Netherlands	September/ October	22.4*	0.16	-	-	64.0	-	32.0	32.0	1.00	-	van Ginneken et al. (2011)
Ulva lactuca	Norway	June	13.3	0.10	26.9	16.3	42.6	1.58	32.1	10.5	0.33	-	Maehre et al. (2014)

* Total Fatty Acids (g/kg DW)

Table 2-12. Essential minerals present in brown seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Alaria esculenta	Norway	May 2010	-	-	-	-	800	870	8.7	4.9	0.56	0.24	22	230	0.004	-	Maehre et al. (2014)
Alaria esculenta	Scotland	March - July 2011	-	-	-	-	-	-	2.33	0.309	0.115	0.031	62.5	-	-	0.011	Schiener et al. (2015)
Ascophyllum nodosum	Canada	September - October 1995	-	-	-	-	-	17.1	3.56	1.91	0.42	482	0.23	-	-	0.016	Phaneuf et al. (1999)
Colpomenia sinuosa	Egypt	April 2011	2451	919	2.67	-	377	114	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Colpomenia sinuosa	Iran	April 2008	424	3510	0.12	-	5227	-	1581	-	9.11	0.94	-	-	-	-	Tabarsa et al. (2012)
Colpomenia sinuosa	Iran	-	-	-	-	-	-	78.1	45.2	1.9	1.5	0.51	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Dictyota dichotoma	Iran	April 2008	634	3417	0.19	-	5257	-	1196	-	8.5	1.29	-	-	-	-	Tabarsa et al. (2012)
Durvillaea antarctica	New Zealand	April 2004	5290	1560	3.39	4149	1410	-	1.37	0.99	0.37	0.509	29.1	773	0.004	0.044	Smith et al. (2010)
Ecklonia radiata	New Zealand	August 2004	3080	5890	0.52	6337	1100	-	28.4	2.07	0.71	1.53	399	1301	0.007	0.261	Smith et al. (2010)
Ecklonia spp.	New Zealand	-	3190	6590	0.48	7313	1160	-	4.25	2.66	0.57	0.181	371.9	-	0.02	0.01	Smith et al. (2010)
Fucus distichus	Canada	September - October 1995	-	-	-	-	-	38.3	3.65	7.46	0.29	212	0.291	-	-	0.026	Phaneuf et al. (1999)

Table 2-12 continued. Essential minerals present in brown seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Fucus serratus	UK	-	-	-	-	-	7.34	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Fucus spiralis	Portugal	January 2013	1429	975.9	1.46	-	118.1	163.2	-	-	-	-	-	-	-	-	Paiva et al. (2014)
Fucus vesiculosis	Spain	-	5469	4322	1.27	-	938	994	4.20	3.71	5.50	-	-	-	-	-	Ruperez (2002)
Fucus vesiculosis	Canada	September - October 1995	-	-	-	-	-	42.7	3.26	15.8	0.41	226	0.316	-	-	0.044	Phaneuf et al. (1999)
Fucus vesiculosis	Norway	May 2010	-	-	-	-	1200	740	9.2	2.6	3.4	0.18	13	84	0.003	-	Maehre et al. (2014)
Himanthalia elongata	Spain	Purchased in 2002	3700	6739	0.55	-	909	826.6	1.81	3.77	4.09	-	-	0.02	<.05	-	Cofrades et al. (2010)
Hormosira banksii	New Zealand	April 2004	5670	4290	1.32	7727	1530	-	23.4	1.27	12.04	1.09	104.1	413	0.015	0.155	Smith et al. (2010)
Laminaria digitata	Spain	-	3818	11579	0.33	-	1005	659	3.29	1.77	-	-	-	-	-	-	Ruperez (2002)
Laminaria digitata	Norway	May 2010	-	-	-	-	1000	840	5.8	2.4	0.31	0.16	310	120	0.002	-	Maehre et al. (2014)
Laminaria digitata	Scotland	August 2010 - October 2011	-	-	-	-	-	-	23.3	3.09	1.15	0.313	624.5	-	-	0.109	Schiener et al. (2015)
Laminaria digitata	UK	-	-	-	-	-	18.2	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)

Table 2-12 continued. Essential minerals present in brown seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Laminaria hyperborea	Norway	May 2010	-	-	-	-	800	640	12	2.2	0.65	0.17	350	160	0.003	-	Maehre et al. (2014)
Laminaria hyperborea	Scotland	August 2010 - October 2011	-	-	-	-	-	-	58.6	2.26	2.38	0.225	81.5	-	-	0.289	Schiener et al. (2015)
Laminaria longicruris	Canada	September - October 1995	-	-	-	-	-	76.3	3.12	0.97	0.15	763	0.601	-	-	0.04	Phaneuf et al. (1999)
Macrocysti spp.	New Zealand	-	4120	11800	0.35	14064	3790	-	26.7	1.893	0.78	0.092	211.6	-	0.001	0.07	Smith et al. (2010)
Padina pavonica	Iran	April 2008	927	2970	0.31	-	3188	-	250	-	10.33	1.21	-	-	-	-	Tabarsa et al. (2012)
Pelvetia canaliculata	Norway	May 2010	-	-	-	-	830	960	13	3.1	0.86	0.26	21	73	0.004	-	Maehre et al. (2014)
Saccharina latissima	Scotland	August 2010 - October 2011	-	-	-	-	-	-	68.3	2.33	2.3	0.267	278.2	-	-	0.276	Schiener et al. (2015)
Saccorhiza polyschides	Portugal	April 2012	2296.2*	7654	0.30	-	911	797	7.9	6.5	0.8	0.3	-	232	-	-	Rodrigues et al. (2015)
Sargassum ilicifolium	Iran	-	-	-	-	-	-	81.7	58.9	2.2	1.6	0.28	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Sargassum linifolium	Egypt	April 2011	2201	1055	2.09	-	144	58	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)

* Sodium content was not declared for these species. Values were calculated from the K content and the Na/K ratio provided.

Table 2-12 continued. Essential minerals present in brown seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Sargassum naozhouense	China	July 2011	3250	4170	0.78	-	66.98	-	147	9.08	5.84	0.36	-	120	-	-	Peng et al. (2013)
Sargassum muticum	Portugal	April 2012	1726.8*	5756	0.30	-	918	1504	19	2.5	1.1	0.5	-	228	-	-	Rodrigues et al. (2015)
Sargassum polycystum	Borneo	-	1362	8371	0.16		3792	487.8	68.2	2.15		0.03	0.766	-	1.14	-	Matanjun et al. (2009)
Undaria pinnatifida	Spain	-	7064	8699	0.81	-	931	1181	7.56	1.74	0.87	-	-	-	-	-	Ruperez (2002)
Undaria pinnatifida	Spain	-	5163	10700	0.48	-	925	833.3	13.3	6.08	0.85	-	-	0.06	<0.05	-	Cofrades et al. (2010)
Undaria pinnatifida	New Zealand	April - September 2004	3610	7120	0.51	8918	1280	-	13.3	2.29	1.01	0.676	17.1	479	0.007	0.074	Smith et al. (2010)
Undaria pinnatifida	New Zealand	-	8720	12300	0.71	20447	860	-	16.8	1.07	0.79	0.079	10.07	-	0.001	0.029	Smith et al. (2010)

* Sodium content was not declared for these species. Values were calculated from the K content and the Na/K ratio provided.

Table 2-13. Essential minerals present in red seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Ahnfeltia plicata	Denmark	September 2011	613	2001	0.31		1151	346	27.3	1.8	17.7	0.41		246	17	0.11	Parjikolaei et al. (2016)
Ceramium spp.	UK	-	-	-	-	-	10.4	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Chondrus crispus	Spain	-	4270	3184	1.34	-	420	732	3.97	7.14	1.32	-	-	-	-	-	Ruperez (2002)
Chondrus crispus	Denmark	September 2011	3059	3256	0.94	-	5398	930	48.8	7.4	65.3	0.51		289	61	0.11	Parjikolaei et al. (2016)
Delesseria sanguinea	Denmark	September 2011	1261	1939	0.65	-	1730	370	71.3	4.9	86.2	1.2		128	34	0.11	Parjikolaei et al. (2016)
Dilsea carnosa	Denmark	September 2011	3311	1304	2.54	-	267	424	10.8	11.2	0.5	0.49		157	-	0.03	Parjikolaei et al. (2016)
Dumontia contorta	UK	-	-	-	-	-	3.87	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Eucheuma cottonii	Borneo	-	1771	13155	0.13	-	329.6	271.3	2.61	4.3	-	0.03	0.942	-	0.59	-	Matanjun et al. (2009)
Furcellaria lumbricalis	Denmark	September 2011	1889	3478	0.54	-	1347	663	46.9	1.6	76.4	0.33	-	84	-	0.07	Parjikolaei et al. (2016)
Gelidium microdon	Azores, Portugal	January 2013	433.0	1238	0.35	-	74.73	127.0	-	-	-	-	-	-	-	-	Paiva et al. (2014)
Gracilaria vermiculophylla	Denmark	September 2011	1017	4912	0.21	-	401	314	35.2	2.4	50.2	0.15		109	-	0.05	Parjikolaei et al. (2016)

Table 2-13 continued. Essential minerals present in red seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Gracilaria edulis	India	-	409.0	90.97	4.50	-	89.378	0.013	0.005	0.001	-	-	-	-	-	-	Sakthivel and Devi (2015)
Gracilaria compressa	Egypt	April 2011	2908	446	6.52	-	333	173	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Gracilaria verrucosa	Egypt	April 2011	2280	817	2.79	-	94	29	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Gracilaria gracilis	Portugal	April 2012	1953*	6510	0.30	-	344	175	9	2.5	2	0.4	-	226	-	-	Rodrigues et al. (2015)
Gracilaria corticata	Iran	-	-	-	-	-	-	18.3	85.0	3.2	3.3	0.33	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Gracilaria salicornia	Iran	April 2008	1036	11380	0.09	-	948	-	67.35	-	4.16	0.57	-	-	-	-	Tabarsa et al. (2012)
Grateloupia turuturu	Portugal	April 2012	960.8	1628	0.59	-	265	695	5	6.9	2.5	0.3	-	281	-	-	Rodrigues et al. (2015)
Hypnea musciformis	Egypt	April 2011	2422	822	2.95	-	379	115	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Hypnea valentiae	Iran	-	-	-	-	-	-	38.7	80.3	3.1	3.7	0.39	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Jania rubens	Egypt	April 2011	1318	283	4.66	-	332	287	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Kappaphycus alvarezzi	India	-	-	-	-	-	-	-	33.8	-	-	-	-	-	-	-	Fayaz et al. (2005)

Table 2-13 continued. Essential minerals present in red seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Kappaphycus alvarezzi	India	September 2004 - April 2006	2230	4100	0.54	-	840	740	65.94	1.85	1.1	0.76	-	120	-	3.88	Kumar et al. (2015)
Mastocarpus stellatus	UK	-	-	-	-	-	8.91	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Odonthalia dentata	Denmark	September 2011	924	2659	0.35	-	1382	342	67.6	2.8	47.9	0.81	-	105	30	0.12	Parjikolaei et al. (2016)
Osmundea pinnatifida	Portugal	January 2013	2669.2	1464.2	1.82	-	411.5	418.6	-	-	-	-	-	-	-	-	Paiva et al. (2014)
Osmundea pinnatifida	Portugal	April 2012	928.8	2610	0.36	-	541	480	37	5.8	1.2	0.5	-	173	-	-	Rodrigues et al. (2015)
Osmundea pinnatifida	UK	-	-	-	-	-	4.426	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Palmaria palmata	Norway	June 2012	-	-	-	-	360	530	10	2.9	1.1	0.49	26	270	0.014	-	Maehre et al. (2014)
Palmaria palmata	Denmark	September 2011	319	4111	0.08	-	933	160	30.7	2.1	57.8	0.47	-	272	-	0.07	Parjikolaei et al. (2016)
Palmaria palmata	USA	-	-	-	-	-	-	-	-	-	-	-	7.2	-	-	-	Teas et al. (2004)
Palmaria palmata	Canada	September - October 1995	-	-	-	-	-	100.9	3.12	3.58	0.81	173	0.094	-	-	0.084	Phaneuf et al. (1999)
Phycodrys rubens	Denmark	September 2011	837	1363	0.61	-	4573	1265	80.1	6	139.2	0.93	-	204	62	0.2	Parjikolaei et al. (2016)

Table 2-13 continued. Essential minerals present in red seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Polysiphonia spp.	UK	-	-	-	-	-	5.37	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Porphyra umbilicais	Spain	-	1173	1407	0.83	-	687	283.3	18.2	4.23	2.72	-	-	0.025	<.05	-	Cofrades et al. (2010)
Porphyra tenera	Japan	-	-	-	-	-	-	-	-	-	-	-	1.6	-	-	-	Teas et al. (2004)
Porphyra tenera	Spain	-	3627	3500	1.04	-	390	565	10.3	2.21	2.72	-	-	-	-	-	Ruperez (2002)
Porphyra columbina	Argentina	August - October 2010	414.2	1444	0.29		443.7	491.5	22	1.46	-	0.51	-	379.9	-	-	Cian et al. (2014)
Porphyra spp.	Portugal	January 2007	2382.6	2481.1	0.96	-	124.5	396.4	-	-	-	-	-	-	-	-	Paiva et al. (2014)
Porphyra spp.	Canada	September - October 1995	-	-	-	-	-	72.4	3.03	3.32	1.03	317	0.19	-	-	0.067	Phaneuf et al. (1999)
Porphyra spp.	New Zealand	May - October 2004	170	2170	0.08	914	850	-	56.9	35.2	1.26	2.362	6.4	547	0.016	0.13	Smith et al. (2010)
Porphyra spp.	New Zealand	-	4240	4370	0.97	8554	210	-	15.89	4.503	1.854	0.424	4.503	-	0.038	0.07	Smith et al. (2010)
Porphyra spp.	UK	-	-	-	-	-	5.16	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Pterocladia capillacea	Egypt	April 2011	1792	832	2.15	-	305	208	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)

Table 2-13 continued. Essential minerals present in red seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Pterocladia capillacea	Egypt	April 2011	1002	942	1.06	-	139	141	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Pterocladia capillacea	Egypt	April 2011	2500	50	50.00	-	385	117	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Pterocladia capillacea	Egypt	April 2011	3966	837	4.74	-	1049	424	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Pterocladiella apillacea	Azores, Portugal	January 2013	635.6	2369	0.27		174	162.7	-	-	-	-	-	-	-	-	Paiva et al. (2014)
Vertebrata lanosa	Norway	June 2012	-	-	-	-	640	600	48	8.1	2	0.8	130	110	0.053	-	Maehre et al. (2014)

Table 2-14. Essential minerals present in green seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Caulerpa lentillifera	Thailand	March	-	970	-	-	780	630	9.3	2.6	7.9	2.2	1.42	1030	-	-	Ratana-arporn and Chirapart (2006)
Caulerpa lentillifera	Borneo	-	8917	1142	7.80	-	1874	1028	21.37	3.51	-	0.11	0.48	-	1.07	-	Matanjun et al. (2009)
Caulerpa racemosa	India	-	22060	1256	17.56	11510	14920	2536	1363	2.51	35	1.47	5.53	571.3	0.11	2.12	Mandlik et al. (2014)
Cladophora rupestris	Norway	May 2010	-	-	-	-	2900	1200	1000	3	24	1.7	6.3	87	0.007	-	Maehre et al. (2014)
Cladophora rupestris	UK	-	-	-	-	-	9.51	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Codium tomentosum	Egypt	April 2011	1179	429	2.75	-	550	83	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Codium tomentosum	Portugal	April 2012	2045	3729	0.55	-	513	1046	28.3	1.8	1.9	0.6	-	180	-	-	Rodrigues et al. (2015)
Enteromorpha spp.	Canada	September - October 1995	-	-	-	-	-	703.7	3.82	15.6	2.27	22.7	0.072	-	-	0.32	Phaneuf et al. (1999)
Enteromorpha spp.	Mexico	Winter 1997	7700	1100	7.00	-	2100	430	-	0.005	-	-	-	2.7	-	-	Aguilera-Morales et al. (2005)
Enteromorpha spp.	Mexico	Winter 1998	9200	1800	5.11		2490	710	-	0.002	-	-	-	3.5	-	-	Aguilera-Morales et al. (2005)
Ulva compressa	Azores, Portugal	April 2013	1322	693.3	1.91		242.6	1594	-	-	-	-	-	-	-	-	Paiva et al. (2014)

Table 2-14 continued. Essential minerals present in green seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Ulva intestinalis	Egypt	April 2011	2212	865	2.56	-	277	281	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Ulva intestinalis	Iran	-	-	-	-	-	-	61.7	25.4	2.1	1.3	0.43	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Ulva intestinalis	Norway	May 2010	-	-	-	-	550	1500	600	2.5	13	0.49	13	120	0.003	-	Maehre et al. (2014)
Ulva intestinalis	Philippines	January - April 2016	-	-	-	-	4.9	-	-	-	-	-	-	-	-	-	Escobido et al. (2016)
Ulva lactuca	Canada	September - October 1995	-	-	-	-	-	248.6	3.33	40.9	1.92	136	0.06	-	-	0.164	Phaneuf et al. (1999)
Ulva lactuca	Egypt	April 2011	2191	770	2.85	-	286	173	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Ulva lactuca	Egypt	April 2011	637	168	3.79	-	647	56	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Ulva lactuca	Egypt	April 2011	2169	723	3.00	-	189	114	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Ulva lactuca	Egypt	April 2011	146	121	1.21	-	234	28	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Ulva lactuca	Egypt	April 2011	837	764	1.10	-	190	144	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Ulva lactuca	Egypt	April 2011	604	145	4.17	-	279	28	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)

Table 2-14 continued. Essential minerals present in green seaweeds.

			Essential Minerals (mg/100g)														
Seaweed	Country	Harvested	Na	K	Na/K Ratio	Cl	Ca	Mg	Fe	Zn	Mn	Cu	I	P	Se	Cr	Author
Ulva lactuca	Egypt	April 2011	333	443	0.75	-	1673	254	-	-	-	-	-	-	-	-	El-Said and El-Sikaily (2013)
Ulva lactuca	Iran	-	-	-	-	-	-	79.1	46.4	1.6	1.5	0.34	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Ulva lactuca	Norway	June 2012	-	-	-	-	350	2600	21	0.8	1.1	0.6	2.1	50	0.005	-	Maehre et al. (2014)
Ulva lactuca	Iran	April 2008	1805	2414	0.75	-	2782	-	199.4	-	2.11	1.45	-	-	-	-	Tabarsa et al. (2012)
Ulva lactuca	UK	-	-	-	-	-	1.99	-	-	-	-	-	-	-	-	-	Marsham et al. (2007)
Ulva lactuca	Ireland	May 2015	1070	1150	0.93	960	2030	2420	35.3	1.7	8.6	2.2	-	256	<0.01	-	Bikker et al. (2016)
Ulva lactuca	Tunisia	July 2007	552	630	0.88	-	2720	3891	41	6.8	1.3	0.8	-	93	-	-	Yaich et al. (2011)
Ulva reticulata	Thailand	May	-	1540	-	-	140	140	174.8	3.3	48.1	0.6	1.124	180	-	-	Ratana-arporn and Chirapart (2006)
Ulva rigida	Spain	-	1595	1561	1.02	-	524.5	2094	283	0.6	1.6	0.5	0.8	210	-	-	Taboada et al. (2010)
Ulva rigida	Azores, Portugal	April 2013	576.0	817.4	0.70	-	324.9	1775	-	-	-	-	-	-	-	-	Paiva et al. (2014)
Ulva stenophylla	New Zealand	April 2004	190	790	0.24	672	1290	-	122.7	6.1	19.23	1.099	2.7	273	0.017	0.173	Smith et al. (2010)

Table 2-15. Vitamin contents of brown seaweeds.

	Vitamin (mg/100g DW)																
Seaweed	Vitamin A	Vitamin D2	Vitamin D3	α-Tocopherol	Δ-Tocopherol	γ-Tocopherol	Vitamin K1	Vitamin K3	Vitamin C	Vitamin B1	Vitamin B2	Vitamin B3	Vitamin B6	Biotin (µg/kg DW)	Folate (g/kg DW)	Vitamin B12 (µg/kg DW)	Author
Ascophyllum nodosum	-	-	-	-	-	-	-	-	81.8	27	7.3	0	0.125	-	0.456	16.4	MacArtain et al. (2007)
Fucus spiralis	1.41	0.21	0.83	51.1	trace	trace	trace	trace	-	-	-	-	-	-	-	-	Paiva et al. (2014)
Laminaria digitata	-	-	-	-	-	-	-	-	355.3	1.38	1.38	612	64.1	-	0	61.9	MacArtain et al. (2007)
Sargassum polycystum	-	-	-	11.3	-	-	-	-	34.5	-	-	-	-	-	-	-	Matanjun et al. (2009)
Undaria pinnatifida	-	-	-	-	-	-	-	-	1847	50.4	117	900	32.4	-	0.066	43.1	MacArtain et al. (2007)

Table 2-16. Vitamin contents of red seaweeds.

	Vitamin (mg/100g DW)																
Seaweed	Vitamin A	β-Carotene	Vitamin D2	Vitamin D3	α-Tocopherol	Δ-Tocopherol	γ-Tocopherol	Vitamin K1	Vitamin K3	Vitamin C	Vitamin B1	Vitamin B2	Vitamin B3	Vitamin B6	Folate (g/kg DW)	Vitamin B12 (µg/kg DW)	Author
Eucheuma cottonii	-	-	-	-	5.85	-	-	-	-	35.3	-	-	-	-	-	-	Matanjun et al. (2009)
Kappaphycus alvarezzi	-	5.26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Fayaz et al. (2005)
Osmundea pinnatifida	1.2	-	trace	-	4.86	trace	14.2	0.92	1.64	-	-	-	-	-	-	-	Paiva et al. (2014)
Palmaria palmata	-	-	-	-	-	-	-	-	-	690	3	10	100	0.25	0.00263	230	MacArtain et al (2007)[S43]
Porphyra umbilicalis	-	-	-	-	-	-	-	-	-	1610.6	9.63	34.3	95.1	14.9	0.125	96.1	MacArtain et al. (2007)
Porphyra spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	250	Watanabe et al. (1999)
Porphyra spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1338	Miyamoto et al. (2009)
Porphyra spp.	1.27	-	0.17	1.05	27.3	1.01	0.18	trace	trace	-	-	-	-	-	-	-	Paiva et al. (2014)

Table 2-17. Vitamin contents of green seaweeds.

	Vitamin (mg/100g DW)											
Seaweed	Vitamin A	α-Tocopherol	Vitamin C	Vitamin B1	Vitamin B2	Vitamin B3	Vitamin B5	Vitamin B6	Biotin (µg/kg DW)	Folate (g/kg DW)	Vitamin B12 (µg/kg DW)	Author
Enteromorpha spp.	-	-	-	-	-	-	-	-	-	-	692	Watanabe et al. (1999)
Caulerpa lentillifera	-	8.41	34.7	-	-	-	-	-	-	-	-	Matanjun et al. (2009)
Ulva rigida	0.29	1.97	94.2	4.7	1.99	< 5	1.7	< 0.1	0.12	1.08	60	Taboada et al. (2010)
Ulva spp.	-	-	1250	7.5	3.75	1000	-	0	-	0.0015	787.5	MacArtain et al. (2007)

Table 2-18. Heavy metal content of brown seaweeds.

			Heavy Metal (mg/100g)									
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Al	Author
Alaria esculenta	Norway	May 2010	4.8	-	0.34	-	-	-	>0.0005	-	-	Maehre et al. (2014)
Alaria esculenta	Scotland	March - July 2011	0.73125	-	-	-	0.002625	-	-	0.01	1.5975	Schiener et al. (2015)
Ascophyllum nodosum	Canada	September - October 1995	-	0.046	0.069	0.086	-	-	-	-	-	Phaneuf et al. (1999)
Colpomenia sinuosa	Iran	-	-	-	-	0.43	-	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Colpomenia sinuosa	Iran	April 2008	-	-	-	0.52	-	-	-	2.58	-	Tabarsa et al. (2012)
Dictyota dichotoma	Iran	April 2008	-	-	-	0.45	-	-	-	2.36	-	Tabarsa et al. (2012)
Durvillae aantartica	Chile	-	1.52	0.0318	0.246	-	-	-	-	-	-	Almela et al. (2006)
Durvillaea antarctica	New Zealand	April 2004	2.713	-	-	-	0.014	-	0.004	-	-	Smith et al. (2010)
Ecklonia radiata	New Zealand	August 2004	5.132	-	-	-	0.061	-	0.017	-	-	Smith et al. (2010)
Ecklonia spp.	New Zealand	-	3.6	0.15	-	-	0.02	-	0.017	-	-	Smith et al. (2010)
Eisenia bicyclis	Japan	-	2.24	0.0167	0.0549	-	0.0169	-	-	-	-	Almela et al. (2006)
Eisenia bicyclis	Japan	-	2.52	0.135	0.0383	-	0.0218	-	-	-	-	Almela et al. (2006)

Table 2-18 continued. Heavy metal content of brown seaweeds.

			Heavy Metal (mg/100g)									
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Al	Author
Eisenia bicyclis	Japan	-	2.63	0.0135	0.0559	-	0.0239	-	-	-	-	Almela et al. (2006)
Eisenia bicyclis	Japan	-	0.41	0.0292	0.0571	-	0	-	-	-	-	Almela et al. (2006)
Eisenia bicyclis	Japan	-	2.66	0.0206	0.0549	-	0	-	-	-	-	Almela et al. (2006)
Fucus vesiculosus	Norway	May 2010	4.1	-	0.12	-	-	-	0.0011	-	-	Maehre et al. (2014)
Fucus spiralis	Portugal	January 2013	-	-	-	-	-	118.1	-	-	-	Paiva et al. (2014)
Fucus vesiculosis	Canada	September - October 1995	-	0.14	0.15	0.12	-	-	-	-	-	Phaneuf et al. (1999)
Fucus distichus	Canada	September - October 1995	-	0.167	0.12	0.119	-	-	-	-	-	Phaneuf et al. (1999)
Himanthalia elongata	Spain	-	2.36	0	0.0389	-	0.0198	-	-	-	-	Almela et al. (2006)
Himanthalia elongata	Spain	-	3.12	0.0202	0.0222	-	0.0126	-	-	-	-	Almela et al. (2006)
Himanthalia elongata	Spain	-	2.13	0	0.0395	-	0.0115	-	-	-	-	Almela et al. (2006)
Himanthalia elongata	Spain	-	3.26	-	<0.04		<0.04	-	-	-	-	Cofrades et al. (2010)
Hizikia fusiforme	Japan	-	11.1	7.54	0.0621	-	0.0885	-	-	-	-	Almela et al. (2006)

Table 2-18 continued. Heavy metal content of brown seaweeds.

			Heavy Metal (mg/100g)									
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Al	Author
Hizikia fusiforme	Japan	-	8.92	4.16	0.107	-	0	-	-	-	-	Almela et al. (2006)
Hizikia fusiforme	Japan	-	11.4	9.12	0.116	-	0	-	-	-	-	Almela et al. (2006)
Hizikia fusiforme	Japan	-	13.1	8.11	0.051	-	0.0537	-	-	-	-	Almela et al. (2006)
Hizikia fusiforme	Japan	-	9.39	6.16	0.116	-	0.006	-	-	-	-	Almela et al. (2006)
Hizikia fusiforme	Japan	-	12.4	8.03	0.0811	-	0	-	-	-	-	Almela et al. (2006)
Hizikia fusiforme	Japan	-	14.9	11.7	0.0948	-	0.0063	-	-	-	-	Almela et al. (2006)
Hizikia fusiforme	Japan	-	6.83	4.37	0.153	-	0.206	-	-	-	-	Almela et al. (2006)
Hizikia fusiforme	Japan	-	10.6	6.94	0.152	-	-	-	-	-	-	Almela et al. (2006)
Hormosira banksii	New Zealand	April 2004	3.169	-	-	-	0.061	-	0.005	-	-	Smith et al. (2010)
Laminaria japonica	Japan	-	11.6	0.144	0.0908	-	0	-	-	-	-	Almela et al. (2006)
Laminaria japonica	Japan	-	10.4	0.0238	0.0074	-	0	-	-	-	-	Almela et al. (2006)
Laminaria digitata	Japan	-	6.57	0.0251	0.0343	-	0.0106	-	-	-	-	Almela et al. (2006)

Table 2-18 continued. Heavy metal content of brown seaweeds.

			Heavy Metal (mg/100g)									
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Al	Author
Laminaria digitata	Norway	May 2010	6.4	-	0.01	-	-	-	0.0006	-	-	Maehre et al. (2014)
Laminaria hyperborea	Norway	May 2010	5.5	-	0.048	-	-	-	0.0007	-	-	Maehre et al. (2014)
Laminaria hyperborea	Scotland	August 2010 - October 2011	6.73	-	-	-	0.116	-	-	0.149	70.29	Schiener et al. (2015)
Laminaria digitata	Scotland	August 2010 - October 2011	7.31	-	-	-	0.0263	-	-	0.1	15.98	Schiener et al. (2015)
Laminaria longicruris	Canada	September - October 1995	-	0.153	0.024	0.147	-	-	-	-	-	Phaneuf et al. (1999)
Macrocysti spp.	New Zealand	-	9.7	0.08	-	-	0.03	-	0.005	-	-	Smith et al. (2010)
Padina pavonica	Iran	April 2008	-	-	-	0.42	-	-	-	2	-	Tabarsa et al. (2012)
Pelvetia canaliculata	Norway	May 2010	2.8	-	0.048	-	-	-	0.0047	-	-	Maehre et al. (2014)
Saccharina latissima	Scotland	August 2010 - October 2011	7.5	-	-	-	0.113	-	-	0.133	102.13	Schiener et al. (2015)
Sargassum naozhouense	China	July 2011	-	-	0.17	-	-	-	-	-	-	Peng et al. (2013)
Sargassum ilicifolium	Iran	-	-	-	-	0.75	-	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Undaria pinnatifida	Japan	-	4.14	0	0.155	-	0.0113	-	-	-	-	Almela et al. (2006)

Table 2-18 continued. Heavy metal content of brown seaweeds.

			Heavy Metal (mg/100g)									
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Al	Author
Undaria pinnatifida	Japan	-	4.52	0	0.102	-	0	-	-	-	-	Almela et al. (2006)
Undaria pinnatifida	Spain	-	4.62	0.112	0.19	-	0.11	-	-	-	-	Almela et al. (2006)
Undaria pinnatifida	Spain	-	2.8	0.0268	0.0227	-	0.0941	-	-	-	-	Almela et al. (2006)
Undaria pinnatifida	Spain	-	3.23	0.0371	0.0.2.	-	0.244	-	-	-	-	Almela et al. (2006)
Undaria pinnatifida	Korea	-	4.6	0.106	0.215	-	0.0648	-	-	-	-	Almela et al. (2006)
Undaria pinnatifida	Japan	-	4.15	0.061	0.122	-	0.0795	-	-	-	-	Almela et al. (2006)
Undaria pinnatifida	Spain	-	3.87	-	<0.04		0.105	-	-	-	-	Cofrades et al. (2010)
Undaria pinnatifida	New Zealand	April - September 2004	3.562	-	-	-	0.023	-	0.003	-	-	Smith et al. (2010)
Undaria pinnatifida	New Zealand	-	3.4	0.01	-	-	0.03	-	0.005	-	-	Smith et al. (2010)

Table 2-19. Heavy metal content of red seaweeds.

			Heavy Metals (mg/100g)								
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Author
Chondrus crispus	Spain	-	1.27	0.0357	0.0722	-	0.0348	-	-	-	Almela et al. (2006)
Chondrus crispus	Spain	-	1.61	0.0842	0.0418	-	0.072	-	-	-	Almela et al. (2006)
Gracilaria corticata	Iran	-	-	-	-	0.81	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Gracilaria edulis	India	-	-	-	-	-	0.0002				Sakthivel and Devi (2015)
Gracilaria salicornia	Iran	April 2008	-	-	-	0.24	-	-	-	0.92	Tabarsa et al. (2012)
Hypnea valentiae	Iran	-	-	-	-	0.45	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Kappaphycus alvarezzi	India	September 2004 - April 2006	-	-	1.00	0.56	-	-	0.05	-	Kumar et al. (2015)
Osmundeap innatifida	Portugal	January 2013	-	-	-	-	-	421.6	-	-	Paiva et al. (2014)
Palmaria palmata	Japan	-	1.26	0.0595	0.0877	-	0.152	-	-	-	Almela et al. (2006)
Palmaria palmata	Canada	September - October 1995	-	0.032	0.049	0.21	-	-	-	-	Phaneuf et al. (1999)
Palmaria palmata	Norway	June 2012	1.00	-	0.048	-	-	-	0.0005	-	Maehre et al. (2014)
Porphyra spp.	Portugal	January 2007	-	-	-	-	-	97.3	-	-	Paiva et al. (2014)

Table 2-19 continued. Heavy metal content of red seaweeds.

			Heavy Metals (mg/100g)								
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Author
Porphyra spp.	Canada	September - October 1995	-	0.029	0.022	0.018	-	-	-	-	Phaneuf et al. (1999)
Porphyra spp.	New Zealand	May - October 2004	1.287	-	-	-	0.098	-	0.003	-	Smith et al. (2010)
Porphyra spp.	New Zealand	-	2.52	0.12	-	-	0.041	-	0.001	-	Smith et al. (2010)
Porphyra tenera	Japan	-	2.41	0.028	0.0089	-	0.0123	-	-	-	Almela et al. (2006)
Porphyra tenera	Japan	-	2.32	0.0167	0.0235	-	0.0126	-	-	-	Almela et al. (2006)
Porphyra tenera	Japan	-	2.41	0.028	0.0089	-	0.0123	-	-	-	Almela et al. (2006)
Porphyra tenera	Japan	-	2.32	0.01667	0.0235	-	0.0126	-	-	-	Almela et al. (2006)
Porphyra umbilicais	Spain	-	3.78	-	<0.04	-	<0.04	-	-	-	Cofrades et al. (2010)
Porphyra umbilicalis	Spain	-	3.45	0.0239	0.0126	-	0.0817	-	-	-	Almela et al. (2006)
Vertebrata lanosa	Norway	June 2012	0.93	-	0.38	-	-	-	0.001	-	Maehre et al. (2014)

Table 2-20. Heavy metal content of green seaweeds.

			Heavy Metals (mg/100g)								
Seaweed	Country	Harvested	As	iAs	Cd	Co	Pb	Cs	Hg	Ni	Reference
Ulva lactuca	Canada	September - October 1995	-	0.022	0.15	0.44	-	-	-	-	Phaneuf et al. (1999)
Enteromorpha spp.	Canada	September - October 1995	-	0.028	0.24	1.05	-	-	-	-	Phaneuf et al. (1999)
Ulva lactuca	Iran	-	-	-	-	0.07	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Ulva intestinalis	Iran	-	-	-	-	0.24	-	-	-	-	Rohani-Ghadikolaei et al. (2012)
Ulva stenophylla	New Zealand	April 2004	0.188	-	-	-	0.183	-	0.01	-	Smith et al. (2010)
Cladophora rupestris	Norway	May 2010	0.94	-	0.0091	-	-	-	0.0006	-	Maehre et al. (2014)
Ulva intestinalis	Norway	May 2010	0.49	-	0.012	-	-	-	0.0014	-	Maehre et al. (2014)
Ulva lactuca	Norway	June 2012	0.79	-	0.0092	-	-	-	0.0005	-	Maehre et al. (2014)
Ulva lactuca	Iran	April 2008	-	-	-	0.15	-	-	-	0.76	Tabarsa et al. (2012)
Caulerpa racemosa	India	-	0.29	-	0.13	<1.3	1.09	5.6	<1	1.15	Mandlik et al. (2014)
Ulva lactuca	Ireland	May 2015	0.58	-	0.0257	0.0271	0.0956		<0.001	0.85	Bikker et al. (2016)
Ulva lactuca	Tunisia	July 2007	-	-	0.12	-	1.26	-	-	-	Yaich et al. (2011)

Chapter 3

Literature Review

Prebiotics from Seaweeds: An Ocean of Opportunity?

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3 Literature Review - Prebiotics from Seaweeds: An Ocean of Opportunity?

3.1 Abstract

Seaweeds are an underexploited and potentially sustainable crop which offer a rich source of bioactive compounds, including novel complex polysaccharides, polyphenols, fatty acids, and carotenoids. The purported efficacies of these phytochemicals have led to potential functional food and nutraceutical applications which aim to protect against cardiometabolic and inflammatory risk factors associated with non-communicable diseases, such as obesity, type 2 diabetes, metabolic syndrome, cardiovascular disease, inflammatory bowel disease, and some cancers. Concurrent understanding that perturbations of gut microbial composition and metabolic function manifest throughout health and disease has led to dietary strategies, such as prebiotics, which exploit the diet-host-microbe paradigm to modulate the gut microbiota, such that host health is maintained or improved. The prebiotic definition was recently updated to "a substrate that is selectively utilised by host microorganisms conferring a health benefit", which, given that previous discussion regarding seaweed prebiotics has focused upon saccharolytic fermentation, an opportunity is presented to explore how non-complex polysaccharide components from seaweeds may be metabolised by host microbial populations to benefit host health. Thus, this review provides an innovative approach to consider how the gut microbiota may utilise seaweed phytochemicals, such as polyphenols, polyunsaturated fatty acids, and carotenoids, and provides an updated discussion regarding the catabolism of seaweed-derived complex polysaccharides with potential prebiotic activity. Additional *in vitro*

screening studies and *in vivo* animal studies are needed to identify potential prebiotics from seaweeds, alongside untargeted metabolomics to decipher microbial-derived metabolites from seaweeds. Furthermore, controlled human intervention studies with health-related end points to elucidate prebiotic efficacy are required.

3.2 Introduction

Seaweeds are an underexploited and sustainable crop which offer a rich source of bioactive compounds, including novel dietary fibres, polyphenols, fatty acids, and carotenoids (Brown et al., 2014; Cherry et al., 2019). Epidemiological evidence comparing Japanese and Western diets have correlated seaweed consumption (5.3 g/day in Japan) with decreased incidence of chronic disease (de Jesus Raposo et al., 2016), while the purported efficacies of seaweed phytochemicals have led to potential functional food and nutraceutical applications which aim to protect against cardiometabolic and inflammatory risk factors associated with non-communicable diseases, such as obesity, type two diabetes, metabolic syndrome, cardiovascular disease, inflammatory bowel disease, and some cancers (Brown et al., 2014).

Current understanding of mutualistic diet-host-microbe interactions has generated efforts to exploit diet to maintain health status, and to prevent or overcome non-communicable diseases, where an imbalance of gut microbiota composition and metabolic function manifests during the onset and pathophysiology of gastrointestinal, neurological, and cardio-metabolic diseases, often congruent with intestinal inflammation and compromised gut barrier function (Schippa and Conte,

2014; Thursby and Juge, 2017). As such, it has become pertinent to explore dietary strategies which modulate gut microbial composition and function to improve host health. This includes the use of prebiotics as fermentable substrates to enable selective gut commensal metabolism.

The prebiotic definition was recently updated to “a substrate that is selectively utilised by host microorganisms conferring a health benefit” (Gibson et al., 2017), which includes the inhibition of pathogens, immune system activation, and vitamin synthesis and provides opportunity to explore the prebiotic efficacy of non-complex polysaccharide components such polyphenols, phytochemicals, and polyunsaturated fatty acids (PUFAs) (Gibson et al., 2017). It is also recognised that other microbial species have the potential to catabolise prebiotics, besides the classical examples of *Bifidobacterium* and *Lactobacillus* (Gibson et al., 2017), courtesy of culture-independent techniques, such as 16S rRNA next generation sequencing and whole genome shotgun metagenomic sequencing which have provided taxonomic classification to identify microbial abundance/diversity and inferred or identified metabolic function (Arnold et al., 2016).

Given that previous discussion regarding the prebiotic potential of seaweed components has focused solely upon the saccharolytic fermentation of complex polysaccharides and the physiological effects of short chain fatty acid metabolites (SCFAs) (de Jesus Raposo et al., 2016; O’Sullivan et al., 2010; Zaporozhets et al., 2014), scope exists to explore the prebiotic potential of other phytochemical components derived from seaweeds, namely polyphenols, carotenoids, and PUFAs, applicable to both human and animal health.

This review aims to provide an updated discussion regarding the fermentation and potential prebiotic effect of seaweed polysaccharides and oligosaccharides, based on recent evidence from *in vitro* fermentation studies and *in vivo* animal models, and to postulate how other seaweed phytochemicals, such as polyphenols, PUFAs, and carotenoids, may interact with the gut microbiota to manipulate microbial composition and/or function to elicit bioactivities pertained to a prebiotic. The latter provides new opportunities to complete prebiotic screening studies using *in vitro* techniques and pre-clinical animal models to understand how parent compound biotransformation into endogenously derived or gut microbiota-derived metabolites impact bioaccessibility and bioavailability to influence gut microbial community structure and function, conducive to a prebiotic effect. Evidence from clinical trials with health-related endpoints and mechanistic insight is imperative to substantiate health claims associated with a prebiotic effect.

3.3 Complex polysaccharides

Seaweeds contain 2.97–71.4% complex polysaccharides (Cherry et al., 2019; de Jesus Raposo et al., 2016), which include alginate, fucoidan, and laminarin in brown seaweeds; xylan and sulphated galactans, such as agar, carrageenan, and porphyran in red seaweeds; whilst ulvan and xylan are found in green seaweeds. The monosaccharide composition of the major brown, red, and green seaweed glycans are presented in **Table 3-1**, **Table 3-2**, and **Table 3-3**, respectively. Whilst no human study to date has explored prebiotic sources from seaweeds, several *in vitro* studies (Bajury et al., 2017; Devillé et al., 2004; Kong et al., 2016; Ramnani et

al., 2012), and *in vivo* animal studies (Devillé et al., 2007; Liu et al., 2015), have explored the prebiotic potential seaweeds and their polysaccharide components.

Seaweed polysaccharides are atypical in structure to terrestrial glycans, and are understood to resist gastric acidity, host digestive enzymes, and gastrointestinal absorption (O'Sullivan et al., 2010). Seaweed glycans may, therefore, serve as fermentation substrates for specific gut microbial populations or facilitate substrate cross-feeding of partially broken-down intermediates, such as oligosaccharides and metabolic cross-feeding of SCFAs to cause indirect proliferation of specific bacteria (Belenguer et al., 2006; Macfarlane and Macfarlane, 2012; Rios-Covian et al., 2017; Rose et al., 2009; Timm et al., 2010). The physiological effects of SCFAs, primarily acetate, propionate, and butyrate, include the reduction of luminal pH to inhibit pathogens, the provision of energy sources to colonocytes, and the activation of free fatty acid receptors; where acetate and propionate are ligands for anorexigenic pathways in appetite regulation and can inhibit the rate limiting step of hepatic cholesterol synthesis via 3-hydroxy-3-methylglutaryl CoA reductase inhibition (Byrne et al., 2015; den Besten et al., 2013; Gunness and Gidley, 2010).

To facilitate saccharolytic fermentation in the colon, the gut microbiota must express functional carbohydrate active enzymes (CAZymes) to catabolise seaweed glycans as carbon sources within the colonic digesta. The repertoire of CAZymes expressed by the human gut microbiota includes glycoside hydrolase and polysaccharide lyase families to facilitate degradation via hydrolysis and elimination reactions, respectively (El Kaoutari et al., 2014; Ndeh and Gilbert, 2018;

Tasse et al., 2010). Whole genome sequencing has previously identified gene clusters which encode the catabolic machinery responsible for the breakdown of prebiotics, which includes the CAZyme families responsible for the catabolism of inulin, lactulose, fructo-oligosaccharides, xylo-oligosaccharides, and galacto-oligosaccharides by human gut commensal strains, including *Bifidobacterium longum* NCC2705, *Bifidobacterium adolescentis* ATCC 15703, *Streptococcus thermophilus* LMD9, *Eubacterium rectale* ATCC 33656, *Bacteroides vulgatus* ATCC 8482, and *Fecalibacterium prausnitzii* KLE1255 (Cecchini et al., 2013).

Based on open source data from the Carbohydrate-Active enZYmes Database (Cantarel et al., 2008), **Table 3-1**, **Table 3-2**, and **Table 3-3** detail the CAZyme families which may exert specificity for seaweed glycans and highlights the gut bacterial populations which have demonstrable evidence for seaweed glycan utilisation. This is dominated by *Bacteroides*, which have extensive glycolytic versatility (Benítez-Páez et al., 2017; Ndeh and Gilbert, 2018). This may explain why *in vitro* batch culture fermentation data of seaweeds and seaweed glycans indicate the proliferation of *Bacteroides*; whilst the degradation of complex seaweed glycans by *Bacteroides* could also facilitate the cross-feeding of oligosaccharides, monosaccharides, and SCFAs for gut commensals deemed beneficial to health, including *Bifidobacterium*.

In vitro fermentation studies are frequently used as screening tools to model colonic fermentation and determine substrate utilisation by an *ex vivo* faecal inoculum, with seaweed as a sole carbon source. An overview of recent *in vitro* fermentation studies which have evaluated the fermentation of whole seaweeds or

extracted complex polysaccharide components by the human gut microbiota is presented in **Table 3-4** (brown seaweeds), **Table 3-5** (red seaweeds), **Table 3-6** (green seaweeds). These tables include differences in study methodologies, for example, test substrate dosage; the use of an *in vitro* digestion before the fermentation experiment (declared within the methods section of the cited research paper); how the inoculum was prepared; duration of the faecal fermentation experiment; microbial enumeration method; and the analytical technique used to ascertain metabolite changes during the fermentation. The use of an *in vitro* digestion before *in vitro* fermentation is often used to determine whether a substrate is resistant to endogenous digestive enzymes and small intestinal absorption, and to provide the fraction of a dietary component which is bioaccessible in the colon (Brodkorb et al., 2019). The lack of an *in vitro* digestion before fermentation experiments may cause false positive results, given that low molecular weight components present in seaweed extracts, normally absorbed in the small intestine, are used as fermentation substrates for the *ex vivo* microbiota. **Table 3-7** highlights data from *in vivo* rodent studies which have evaluated the potential prebiotic effect of seaweeds and seaweed glycans.

3.3.1 Brown seaweed polysaccharides

Brown seaweeds are commonly used as food ingredients owing to their commercial abundance (Usman et al., 2017). The anti-obesogenic effects of brown seaweeds are reported in mice, where supplementation of 5% (*w*/*w*) *Saccorhiza polyschides* extract, containing 12% dietary fibre, reduced body weight gain and fat mass of mice with diet-induced obesity (Huebbe et al., 2017). The anti-obesogenic effect was attributed to the fermentation of alginate and fucoidan complex

polysaccharide components, owing to reduced microbial bile salt hydrolase activity; however, no gut microbial compositional data were provided. Elsewhere, the *in vitro* evidence (**Table 3-4**) indicates that whole brown seaweeds and their extracted complex polysaccharide components are fermented by the *ex vivo* faecal microbiota, with increased production of acetate, propionate, butyrate, and total SCFAs reported during fermentation experiments. A corresponding increase in populations, such as *Bifidobacterium, Bacteroides*, *Lactobacillus, Roseburia*, *Parasutterella, Fusicatenibacter*, *Coprococcus, Fecalibacterium* is also reported (Charoensiddhi et al., 2017b, 2016; Fu et al., 2018).

3.3.1.1 Alginate

Alginates are composed of 1,4-linked α-l-guluronic (G) and β-d-mannuronic acid (M) residues to form GM, GG and MM blocks, and represent 17–45% dry weight of brown seaweeds (Vera et al., 2011). The colloidal properties of alginates have wide application in food processing, biotechnology, medicine and pharmaceutical industries (García-Ríos et al., 2012), while degraded sodium alginate is an approved item of “foods with specified uses”, under the categories of “Foods that act on cholesterol plus gastrointestinal conditions” and “Foods that act on blood cholesterol levels” in Japan (Maeda-Yamamoto, 2017). The presence of water soluble alginate oligosaccharides in the faeces of pigs fed alginate is indicative of alginate lyase activity by the luminal or mucus adherent gut microbiota (Jonathan et al., 2013), although an adaptation period of > 39 days is reported for the degradation of G blocks by the porcine microbiota whilst M blocks are readily degraded (Jonathan et al., 2015).

The capacity for alginate to modulate the gut microbiota of Japanese individuals was highlighted over 20 years ago (Terada et al., 1995), where alginate supplementation (30 kDa, 10 g/day, n = 8) significantly increased faecal *Bifidobacterium* populations in healthy male volunteers after both one and two weeks, alongside significantly increased acetic and propionic acids after two weeks. Deleterious metabolites, including faecal sulphide, phenol, p-cresol, indole, ammonia and skatole were significantly reduced compared to the control (free living) diet. Notably, faecal *Bifidobacterium* counts and SCFA concentrations returned to baseline in the week after alginate diet cessation, which highlights the transient nature of the gut microbiota and the need for greater powered long-term human intervention studies.

Subsequent *in vitro* fermentation studies have indicated that alginate is fermented by the human gut microbiota, for example, a 24 h *in vitro* fermentation of a 212 kDa alginate increased total bacterial populations, although no statistical increase in individual *Bifidobacterium*, *Bacteroides*/*Prevotella*, *Lactobacillus*/*Enterococcus*, *Eubacterium rectale*/*Clostridium coccoides*, or *Clostridium histolyticum* populations were observed (Ramnani et al., 2012). Acetic acid, propionic acid and total SCFAs were significantly increased after 24 h fermentation with the 212 kDa alginate, while alginate of 97 kDa increased total SCFA and acetate production after 10 h of fermentation. Alginates of 38 kDa, and 97 kDa did not change microbial abundance, although the authors could not correlate molecular weight with fermentation patterns.

Alginate oligosaccharides (AOS) (~3.5 kDa) can be obtained via acidic or enzymatic hydrolysis of alginate polysaccharides (Vera et al., 2011), and enzymatically derived AOS has promoted the growth of *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium longum* SMU 27001 and lactobacilli, *in vitro* (Wang et al., 2006). Supplementation of 2.5% AOS for two weeks significantly increased faecal *Bifidobacterium*. in rats compared to control and 5% FOS supplemented diets (13-fold and 4.7-fold increase, respectively), while faecal *Lactobacillus* were 5-fold greater in rats who consumed AOS compared to FOS. *Enterobacteriaceae* and *Enterococcus* populations were significantly decreased following AOS supplementation. Elsewhere, the hydrolysis of alginate, mannuronic acid oligosaccharides (MO) and guluronic oligosaccharides (GO) during a 48 h batch culture fermentation with the faecal microbiota of Chinese individuals demonstrated increased production of acetate, propionate, butyrate, and total SCFAs compared to the substrate-free control, where GO generated the greatest increase (Li et al., 2016). Subsequent strain isolation from the stools of individuals who demonstrated alginate degradation during fermentation identified *Bacteroides xylanisovlens* G25, *Bacteroides thetaiotomicron* A12, *Bacteroides ovatus* A9, and *Bacteroides ovatus* G19 as strains capable of hydrolysing alginate and AOS, where *Bacteroides ovatus* G19 expressed α-1,4-guluronanlyase and β-1,4-mannuronanlyase CAZymes (Li et al., 2017).

A *Bacteroides xylanisolvens* strain with 99% similarity to *Bacteroides xylanisolvens* XB1A was recently isolated from the stool of a Chinese individual and the alginate lyase gene expressed was 100% homologous to the alginate lyase of *Bacteroides ovatus* strain ATCC 8483 (Bai et al., 2017). The preceding *in vitro* fermentation

study demonstrated increased production of acetate, propionate, butyrate, and total SCFAs compared to the soluble starch control vessel following a 72 h fermentation of alginate.

Alginate lyase depolymerises alginate polysaccharides to lower molecular weight oligosaccharides via β-elimination, and is most commonly expressed by marine bacteria, including *Flammeovirga*, *Vibrio*, *Pseudoalteromonas*, *Glaciecola chathamensis* S18K6, and *Zobellia galactanivorans* (Chen et al., 2016; Dong et al., 2017; Han et al., 2016; Xu et al., 2017; Zhu et al., 2018), while the terrestrial bacteria *Paenibacillus* sp. Strain MY03 was recently reported to possess genes encoding alginate lyase and agarase enzymes (Liu et al., 2017). The acquisition of genes encoding alginate lyase enzymes by human gut *Bacteroides* is a suggested consequence of horizontal gene transfer from the marine environment (Mathieu et al., 2016; Thomas et al., 2012), where seaweed consumption may have provided a vector to exert a selective pressure to induce diet-driven adaptations of the gut microbiota (Bhattacharya et al., 2015; Cantarel et al., 2012; Hehemann et al., 2014; Martin et al., 2015; Mathieu et al., 2018; Singh and Reddy, 2016). Recent work by Matthieu *et al.* (Mathieu et al., 2018) suggests that an alginate degradation system within the genome of human gut *Bacteroides* was a result of ancient acquisition, where the polysaccharide utilisation loci encodes PL6 and PL17 alginate lyase enzymes and hypothetical proteins responsible for alginate recognition, internalisation, and catabolism, including bacterial ABC transporter proteins to facilitate alginate uptake across the bacterial membrane (Maruyama et al., 2015). Nevertheless, *in vivo* rodent studies have demonstrated that seaweed glycans are fermented even though animals have never been exposed to dietary seaweeds

before the intervention, which suggests that the gut microbiome contains genes for CAZymes which can degrade seaweed glycans when expressed.

3.3.1.2 Laminarin

Laminarin is a water-soluble storage polysaccharide consisting of 1,3- or 1,6-β-glucose with an average molecular weight of 5 kDa (Kadam et al., 2015) and accounts for 10–35% of the dry weight of brown seaweeds (Vera et al., 2011). One *in vitro* batch culture fermentation of laminarin demonstrated increased *Bifidobacterium* and *Bacteroides* after 24 h (Seong et al., 2019), while another demonstrated increased propionate and butyrate production after 24 h (Devillé et al., 2007). A subsequent *in vivo* rat study (143 mg laminarin per kg body weight per day for 14 days) indicated that laminarin was not selectively fermented by *Lactobacillus* and *Bifidobacterium*, but could modify jejunal, ileal, caecal and colonic mucus composition, secretion, and metabolism to protect against bacterial translocation. The authors suggest that increased luminal acidity and/or catabolism of laminarin by mucolytic commensals could elicit such effects, which corroborates the evidence that a complex polysaccharide-rich diet maintains mucus layer integrity to promote gut barrier function (Brownlee et al., 2003; Desai et al., 2016). Future studies regarding intestinal mucus modulation by laminarin may wish to characterise gut microbiota compositional and functional changes following laminarin ingestion, to detect the abundance and metabolic activity of glycan degraders, such as *Bacteroides* (Salyers et al., 1977; Salyers et al., 1977) or mucolytic species associated with health, such as *Akkermansia muciniphila* or *Ruminococcus* (Dao et al., 2016; Tailford et al., 2015). Elsewhere, laminarin increased L-cell GLP-1 secretion to attenuate diet-induced obesity in mice, and

improved glucose homeostasis and insulin sensitivity (Yang et al., 2017). The authors suggested that the observed cytosolic Ca^{2+} cascade caused GLP-1 secretion, which is in agreement with GPR41/43 receptor activation by SCFAs produced by gut microbial fermentation (Everard and Cani, 2014; Tolhurst et al., 2012), however, data obtained to assess laminarin-induced changes to gut microbiota composition and metabolic output is needed to ascribe a prebiotic effect in this study.

The abundance of glycoside hydrolase and β-glucosidase enzymes expressed by the human gut microbiota may have the capacity to catabolise laminarin (Dabek et al., 2008; Gloux et al., 2011; Michalska et al., 2013; Tasse et al., 2010), for example, a *Bacteroides cellulosyliticus* WH2 human gut isolate was able to grow on laminarin-supplemented minimal media *in vitro*, (incidentally it did not grow on alginate, carrageenan, or porphyran) (McNulty et al., 2013); however, the molecular mechanisms by which human gut *Bacteroides* breakdown laminarin are likely distinct from those responsible for the degradation of mix linked β 1,3- 1,4-glucans, such as those found in cereals (e.g., by $BoGH16_{MLG}$) (Tamura et al., 2017).

3.3.1.3 Fucoidan

Fucoidans are water soluble polysaccharides composed of sulphated 1,2- or 1,3- or 1,4-α-l-fucose which exist as structural polysaccharides in brown seaweeds and occupy 5–20% of algal dry weight (Li et al., 2008; Vera et al., 2011). The structural heterogeneity of fucoidan encompasses varying degrees of branching, sulphate content, polydispersity, and irregular monomer patterns, which can include fucose,

uronic acid, galactose, xylose, arabinose, mannose, and glucose residues (García-Ríos et al., 2012; Jiao et al., 2011; Zaporozhets et al., 2014).

A recent *in vitro* fermentation study of fucoidan (< 30 kDa) extracted from *Laminaria japonica* demonstrated a greater increase in *Bifidobacterium* and *Lactobacillus* following 24 h and 48 h fermentation relative to > 30 kDa fucoidan (Kong et al., 2016), while fucoidan from *Ascophyllum nodosum* (1330 kDa) and *Laminaria japonica* (310 kDa) were shown to increase *Lactobacillus* and *Ruminococcaceae*, respectively, in the caecal microbiota of mice gavaged with 100 mg/kg/day (Shang et al., 2016). Fucoidan also reduced serum LPS-binding protein levels in this study—indicative of a reduced antigen load and reduced inflammatory response. In contrast, fucoidan with a fucose-rich and highly sulphated fucoidan extracted from *Cladosiphon okamuranus* was not fermented by the rat gut microbiota (Choa An et al., 2013).

While the purported bioactivities of fucoidan include anti-obesogenic, anti-diabetic, anti-microbial, and anti-cancer properties (Collins et al., 2016), there is limited evidence to implicate a role for the gut microbiota with such bioactivities, and studies are needed to evaluate the structure-dependent fermentation of fucoidan to ascribe a prebiotic effect. For the latter, this is surprising given the myriad of α-fucosidase enzymes present in the human gut bacterial glycobiome.

3.3.2 Red seaweed polysaccharides

3.3.2.1 Galactans (Carrageenan, Agar, and Porphyran)

Red seaweeds, such as *Gelidium* spp. and *Gracilaria* spp., are used in the commercial production of agar and carrageenan food additives, including

thickening, stabilizing and encapsulation agents (Usman et al., 2017). A summary of evidence from recent *in vitro* fermentation experiments using red seaweed-derived substrates are presented in **Table 3-5**.

Carrageenans are composed of sulphated 1,4-β-d-galactose, 1,3-α-d-galactose, and 3,6-anhydro-d-galactose (Weiner, 2014), and constitutes 30−75% dry weight of red seaweeds (Vera et al., 2011). In rats fed 2.5% *Chondrus crispus*, of which carrageenan is a major polysaccharide component, faecal *Bifidobacterium breve, and a*cetate, propionate, and butyrate SCFAs *were significantly increased alongside a significant decrease in the* pathogens *Clostridium septicum* and *Streptococcus pneumonia,* as compared to the basal diet (Liu et al., 2015). Furthermore, a 1:1 mixture of polysaccharide extracts from *Kappaphycus alvarezii* (containing carrageenan) and *Sargassum polycystum* (brown seaweed) has lowered serum lipids in rats (Dousip et al., 2014). In a study by Li et al. (Li et al., 2017), β-carrageenase activity in a *Bacteroides uniforms* 38F6 isolate complex of *Bacteroides xylanisolvens* and *Escherichia coli* hydrolysed κ-carrageenan oligosaccharides into 4-*O*-sulfate-d-galactose, κ-carratriose, κ-carrapentaose, and κ-carraheptaose, which could facilitate cross-feeding to promote the growth of *Bifidobacterium* populations.

Agar is composed of sulphated 1,3-β-d-galactose and 1,4- 3,6-anhydro-α-l-galactose (Lahaye and Rochas, 1991) and can be fractionated into agarose and agaropectin (O'Sullivan et al., 2010). Low molecular weight agar of 64.64 kDa has demonstrated a bifidogenic effect alongside increased acetate and propionate SCFA concentrations after 24 h *in vitro* fermentation with human stool inoculum

(Ramnani et al., 2012), while mice fed with 2.5% (*w*/*v*) neoagarose oligosaccharides for 7 days demonstrated increased caecal and faecal *Lactobacillus* and *Bifidobacterium* (Hu et al., 2006). The utilisation of agaro-oligosaccharides was noted *in vitro* by *Bacteroides uniforms* L8, isolated from Chinese individuals, which secreted a β-agarase CAZyme to breakdown agarooligosaccharides into agarotriose and subsequently facilitated the growth of *Bifidobacterium infantis* and *Bifidobacterium adolescentis* via the cross feeding of agarotriose (Li et al., 2014).

Porphyran is made up of sulphated 1,3-β-d-galactose, 1,4-α-l-galactose-6-sulfate and 3,6-anhydro-α-l-galactose (Hehemann et al., 2010; Muraoka et al., 2008; Zhang et al., 2004). An *in vitro* faecal fermentation study indicated that porphyran did not significantly increase SCFAs, but stimulated *Lactobacillus* and *Bacteroides* populations (Seong et al., 2019). While pure cultures of *Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium adolescentis,* but not *Bifidobacterium bifidum*, were able to ferment dried *Porphyra yezoensis* (Nori), containing a low protein content (25%), whereas Nori with a high protein content (41%) was not fermented (Muraoka et al., 2008). It is likely that carbohydrate content was highest in the low protein Nori, thus seasonal- and species-variation and in seaweed macronutrient content should be considered a determinant factor for the fermentability of whole seaweeds (Kravchenko et al., 2018; Medcalf et al., 1975; Rioux et al., 2009; Skriptsova, 2016).

Evidence for the horizontal transfer of genes for porphyranase and agarase CAZymes from the marine bacteria, *Zobellia galactanivorans*, to *Bacteroides plebeius* of Japanese individuals is indicative of diet-driven adaptations of the

human gut microbiome (Hehemann et al., 2012, 2010); however, the North American counterparts in this study did not consume seaweeds and the gut microbiota of these individuals did not express such CAZymes. This may mean that the fermentation of seaweed polysaccharides, such as porphyran and agar, requires exposure to, and acquisition of, specific CAZymes usually present in the marine environment (Hehemann et al., 2014). Red seaweed galactans are emerging prebiotic candidates given the commercial availability of red seaweed hydrocolloids and the potential gut modulatory effects of oligosaccharides obtained from red seaweeds. Nevertheless, further *in vivo* evidence is needed, given the purported pro-inflammatory effects of low molecular weight carrageenan (Bhattacharyya et al., 2010; Shang et al., 2017; Younes et al., 2018).

3.3.2.2 Xylan

Xylan, composed of 1,3-1,4-β-D-xylose, is a major constituent of red seaweeds, such as *Palmaria palmata* (Usov, 2011). A previous *in vitro* faecal fermentation study of xylan derived from *P. palmata*, reported that xylose was fermented after six hours alongside a 58:28:14 ratio of acetate, propionate, and butyrate SCFAs (total SCFAs were 107 mM/L) (Lahaye et al., 1993). This study did not ascertain bacterial compositional data, and thus a knowledge gap is presented given that xylans and xylooligosaccharides (XOS) extracted from terrestrial plants, such as wheat husks and maize, are mooted as potential prebiotics owing to evidence of bifidogenesis, improved plasma lipid profile, and positive modulation of immune function markers in healthy adults (Childs et al., 2014; Lecerf et al., 2012). Given that human gut *Bacteroides* express a repertoire of xylanase and xylosidase

CAZymes (Mirande et al., 2010), investigations regarding the capacity of the human gut microbiota to catabolise red seaweed xylans and XOS are suggested.

3.3.3 Green seaweed polysaccharides

3.3.3.1 Ulvan

Ulvans are water-soluble cell wall polysaccharides that account for 8–29% dry weight of green seaweeds, and are composed of sulphated 1,3-α-L-rhamnose, 1,4-β-D-glucuronic acid, and 1,4-β-D-xyloglucan (Lahaye and Robic, 2007). Previous reports indicate that *Ulva lactuca* and ulvan polysaccharides are poorly fermented by the human gut microbiota (Andrieux et al., 1998; Jiao et al., 2011; O'Sullivan et al., 2010), while an *in vitro* fermentation study of *Enteromorpha* spp. with a human faecal inoculum reported no difference in *Enterococcus, Lactobacillus*, and *Bifidobacterium* populations compared to the control; only an increase in *Enterobacter* after 24 h and 48 h of fermentation (**Table 3-6**) (Kong et al., 2016). In contrast, a recent *in vitro* faecal fermentation study indicated that Ulvan stimulated the growth of *Bifidobacterium* and *Lactobacillus* populations and promoted the production of lactate and acetate (Seong et al., 2019). Further, a murine study showed that *Enteromorpha* (EP) and *Enteromorpha* polysaccharides (PEP) ameliorated inflammation associated with Loperamide-induced constipation in mice (Ren et al., 2017), where alpha diversity, Firmicutes, and Actinobacteria were increased in the faecal microbiota of seaweed-supplemented mice compared to the constipated control. Bacteroidetes and Proteobacteria were decreased, while Bacteroidales family *S24-7* and *Prevotellaceae* were increased in EP and PEP, respectively. Current evidence for the fermentation of green seaweeds and their

polysaccharides is limited and fermentation may require specific α-L-rhamnosidase activity by gut commensals (Munoz-Munoz et al., 2017). More experimental evidence is needed to understand the impact of ulvans and ulvan-oligosaccharides in the human and animal diet.

3.3.4 Future prospective – Obtaining oligosaccharides

Enzyme technologies are reported to increase the extraction yield and reduce the molecular weight of bioactive components obtained from seaweeds, with examples of enhanced prebiotic activity when commercially available cellulases or seaweed-specific enzymes were used to hydrolyse polysaccharides (Charoensiddhi et al., 2017a; Rodrigues et al., 2016). Despite limited commercial availability of seaweed-specific enzymes, an avenue for functional oligosaccharide production is presented if efforts to develop commercially viable saccharolytic enzymes from microorganisms (primarily marine). Examples of such glycoside hydrolases include fucoidanase from *Sphingomonas paucimobilis* PF-1 (Kim et al., 2015); ulvan lyase from *Alteromonas* spp. (Coste et al., 2015) and the family *Flavobacteriaceae* (Thomas et al., 2012); β-agarase from *Cellulophaga omnivescoria* W5C (Ramos et al., 2018) and *Cellvibrio PR1* (Xie et al., 2017); alginate lyase from *Flammeovirga* (Cheng et al., 2017), and *Paenibacillus* (Liu et al., 2017); and laminarinase from *Clostridiium thermocellum* (Kislitsyn et al., 2015). Factors which influence the stability and efficacy of such hydrolytic enzymes may include metal ion interaction, or thermostability at the high temperatures needed to prevent gelling of polysaccharides. Recent insight into the production of agarose oligosaccharides and neoagarose oligosaccharides from agar exemplify this (Xu et al., 2018).

3.4 Polyphenols

Seaweeds are rich in polyphenols, such as catechins, flavonols, and phlorotannins. Red and green seaweeds are a source of bromophenols, phenolic acids, and flavonoids (Gómez-Guzmán et al., 2018), while phlorotannins are the most abundant polyphenol in brown seaweeds. Most research to date concerns the bioactivity of phlorotannins, a class of polyphenol unique to brown algae comprised of phloroglucinol monomers and categorised as eckols, fucols, fuhalols, ishofuhalols, phloroethols, or fucophloroethols (Gómez-Guzmán et al., 2018). The purported bioactivities of seaweed polyphenols are associated with the mitigation of risk factors pertained to type 2 diabetes and cardiovascular disease, including hyperglycemia, hyperlipidemia, inflammation and oxidative stress (Lee et al., 2012; Lopes et al., 2017; Murray et al., 2018; Murugan et al., 2015; Shanura et al., 2016), and also anti-microbial activity (Eom et al., 2012). Owing to heterogeneity in both molecular weight and the level of isomerisation, characterisation of polyphenols is difficult (Heffernan et al., 2015; Melanson and MacKinnon, 2015; Montero et al., 2016), and a paucity of information exists regarding the endogenous digestion and microbial catabolism of seaweed polyphenols, with a scarce mechanistic understanding of how they may exert health benefits via the gut microbiota.

Most polyphenols of plant origin must undergo intestinal biotransformation by endogenous enzymes and the gut microbiota prior to absorption across enterocytes. These enzymatic transformations include the elimination of glycosidic bonds, for example, flavonoids are converted to glycones (sugars) and aglycones (non-sugars–polyphenols) by endogenous β-glucosidases in the small intestine

(Lewandowska et al., 2013). The transport of aglycones to the liver via the portal vein results in phase II biotransformation (coupling reactions, chiefly hepatic conjugation to O-glucuronides and O-sulfates) to facilitate urinary and biliary elimination. Phase II metabolites are absorbed into the systemic circulation, or excreted in bile and re-enter the duodenum (hepatic recycling), where subsequent glucuronidase, glycosidase, or sulphatase-mediated deconjugation by the colonic microbiota may favour aglycone reabsorption (Opara and Chohan, 2014).

Approximately 90–95% of dietary polyphenols reach the colon intact (Clifford, 2005), where biotransformation and metabolism by the gut microbiota occurs via hydrolysis, reduction, decarboxylation, demethylation, dehydroxylation, isomerisation, and fission (Selma et al., 2009), to produce low-molecular weight compounds with less chemical heterogeneity than the polyphenol parent compound (Lewandowska et al., 2013). It is suggested that a complex network of gut microbial species is necessary for full biotransformation of polyphenols, whereas simple reactions, such as deglycosylation, can be achieved by individual gut strains. Furthermore, the bioactivities associated with dietary polyphenol intake may be dependent on the catabolic capacity and composition of the gut microbiota, owing to the biological activity of metabolites rather than the parent polyphenol compound present in food (Espín et al., 2017; Williamson and Clifford, 2017), while a synergistic effect between prebiotic polyphenols and probiotic bacteria may occur (Gibson et al., 2017).

The identification of bacteria which possess the metabolic capabilities to utilise polyphenols was previously identified in *Eubacterium oxidoreducens*, which could

catabolise gallate, pyrogallol, phloroglucinol and quercetin (Krumholz and Bryant, 1986). Quercetin biotransformation by *Eubacterium ramulus* has also been identified (Schneider and Blaut, 2000), and multiple human gut microbes which possess phenolic enzymes capable of breaking down glycosides, glucuronides, sulphates, esters, and lactones was summarised by Selma *et al.* (Selma et al., 2009). Such microorganisms included *E. coli* with β-glucuronidase activity; *Eubacterium*, *Bacteroides*, and *Clostridium* with β-glucosidase activity; *Lactobacillus*, *Eubacterium*, *Clostridium*, *Butyrbacterium*, *Streptococcus*, and *Methylotrophicum* with demethylase activity; and *E. coli*, *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Streptococcus, Ruminococcus*, and *Enterococcus* with esterase activity. There is also evidence for α-l-Rhamnosidase mediated hydrolysis of rutinose, present on glycosylated polyphenols (rhamnoglycosides), to produce aglycones, by species, such as *Bacteroides thetaiotaomicron* (Munoz-Munoz et al., 2017), *Bifidobacterium dentium* (Bang et al., 2015), *Bifidobacterium catenulatum* (Amaretti et al., 2015), *Bifidobacterium pseudocatenulatum* (Amaretti et al., 2015), and *Lactobacillus plantarum* (Delgado et al., 2017).

Current knowledge regarding the fate of seaweed polyphenols in the human gastrointestinal tract is scarce; however, it is understood that the limited absorption of *Ascophyllum nodosum* polyphenols from small intestinal enterocytes to the portal vein may facilitate the conjugation of polyphenols to methylated, glucuronidated, or sulphated forms rather than hydrolysis to aglycones (Corona et al., 2017, 2016). Subsequently, unabsorbed conjugated polyphenols are available for biotransformation by the colonic microbiota, then potentially absorbed across the colonocytes. Indeed, Corona *et al*. (Corona et al., 2017), observed a reduction

of total polyphenol contents of an *Ascophyllum nodosum* polyphenol extract, high molecular weight fraction (> 10 kDa), and low molecular weight fraction (1–10 kDa) following *in vitro* digestion and batch culture fermentation; although anti-genotoxic activity against H_2O_2 induced DNA damage of HT-29 cells was increased (to a greater extent by the high molecular weight fraction). This study did not assess the microbiota composition, however, elsewhere, an *in vitro* fermentation of an *Ecklonia radiata* phlorotannin extract significantly increased *Bacteroidetes*, *Clostridium coccoides*, *E. coli*, and *Fecalibacterium prausnitzii*, but decreased *Bifidobacterium* and *Lactobacillus* populations after 24 h fermentation (Charoensiddhi et al., 2017c). More *in vitro* digestion studies would be useful to understand the stability of seaweed polyphenols as extracts or within the seaweed matrix (Oliveira and Pintado, 2015; Sadeghi Ekbatan et al., 2016). These studies may be complemented by studies which use ileostomy patient cohorts to determine structural changes to seaweed polyphenols following upper GI digestion *in vivo* to indicate polyphenol bioaccessibility in the colon (Brown et al., 2014).

A recent review highlighted the potential for dietary polyphenols to modulate the gut microbiota by increasing *Bifidobacterium, Lactobacillus, Bacteroides*, *Enterococcus, Akkermansia muciniphila*, and *Fecalibacterium prausnitzii* populations (Duenas et al., 2015). This review did not include any studies which assessed modulation of the gut microbiota by seaweed polyphenols and, therefore, a research opportunity is presented. Inter-individual variation of gut microbiota composition and function is the key determinant for gut microbiota-mediated biotransformation of phenolic compounds to bioactive metabolites (Duda-Chodak et al., 2015; Tomás-Barberán et al., 2016). Therefore, identification

of bacterial species or strains with the ability to catabolise seaweed polyphenols and their respective catabolic machinery is needed to understand if seaweed polyphenols could be prebiotic (Núñez-Sánchez et al., 2015; Ozdal et al., 2016). Moreover, considering that gut microbiota-derived secondary metabolites reach a peak plasma concentration much later than the original aglycone or hepatic conjugates, controlled nutrikinetic studies could elude how dietary polyphenols from seaweeds interact with host-microbiota metabolism (Laura et al., 2014; van Duynhoven et al., 2011). Identification of faecal, urinary, serum, or tissue biomarkers via untargeted and targeted metabolomics approaches, and the use of stable isotope studies, may also indicate variation in synthesis, bioavailability, metabolism, and excretion of polyphenols and associated metabolites. While integration of dose response studies alongside metagenomics and metabolomics analyses, akin to those conducted for berry and wine polyphenols, could elude how much seaweed polyphenol is required to have an impact, if any, on gut microbial composition, metabolic function, and host health (Duenas et al., 2015; Feliciano et al., 2017).

3.5 Other seaweed phytochemicals

3.5.1 Carotenoids

Carotenoids are lipid soluble compounds which function within the photosynthetic machinery of seaweeds to produce pigments. Fucoxanthin is the predominant carotenoid in brown seaweeds (Rajauria et al., 2017), while lutein, β-carotene, astaxanthin, echinenone, violaxanthin, neoxanthin, and zeaxanthin are found in red and green seaweeds. Carotenoids are used as food colouring additives, while

the application of fucoxanthin as functional food ingredients is suggested, owing to putative anti-oxidant, anti-inflammatory, anti-cancer, anti-obesity, and anti-diabetic bioactivities (Christaki et al., 2013; Kulczyński et al., 2017; Lopes-Costa et al., 2017; Maeda et al., 2008; Mikami and Hosokawa, 2013; Woo et al., 2010).

While some carotenoids are absorbed by enterocytes and converted into vitamin A and retinoid derivatives by endogenous beta-carotene oxygenase 1 (BCO1) and beta-carotene dioxygenase 2 (BCO2) enzymes (Bohn et al., 2015; Widjaja-Adhi et al., 2015), the bioavailability of carotenoids in the blood is reported as 10–40% (Rein et al., 2013), which has led to suggestions that carotenoids could be fermented by the gut microbiota (Bohn, 2018; Bohn et al., 2015). The only evidence to date has demonstrated that male C57BL/6J mice supplemented with 0.04% (*w*/*w*) astaxanthin during an eight-week pilot study had increased abundance of caecal *Bifidobacterium* (Lyu et al., 2018), whereas *Proteobacteria* and *Bacteroides* were significantly increased in the caecum of BCO2 knockout mice; however, analysis of health biomarkers was not reported. Given the differences in microbiota composition between wild type and BCO2 knockout mice in this study, there is scope to investigate how carotenoids and their endogenous derivatives interact with the gut microbiota. Looking ahead, the use of *in vitro* models of gastrointestinal digestion and colonic fermentation would be useful to assess whether there is a direct substrate to microbiota effect or a host–microbe effect (Kamiloglu and Capanoglu, 2018).

3.5.3 Polyunsaturated fatty acids (PUFAs)

The lipid content of seaweed ranges from 1–5% dry weight, which includes n-3 PUFAs, such as eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) (Robertson et al., 2015; van Ginneken et al., 2011). The n-3 PUFA are associated with the anti-inflammatory activity to reduce cardiovascular disease risk and may also exert beneficial effects on brain function and behaviour, as mediated by the microbiota-gut-brain axis (Costantini et al., 2017). Dietary EPA and DHA intake are reported to improve microbial diversity, reduce the Firmicutes/Bacteroidetes ratio, reduce LPS-producing bacteria, and increase populations of *Bifidobacterium*, *Lachnospiraceae*, and lipopolysaccharide (LPS)-suppressing bacteria in both humans and animal models (Costantini et al., 2017; Menni et al., 2017; Robertson et al., 2018). Although the evidence to date has focused on fish-derived n-3 PUFA, great scope exists to evaluate the prebiotic effect of n-3 PUFA obtained from seaweeds.

3.6 Fermented foods

Fermented foods are understood to have improved nutritional and functional properties owing to bioactive or bioavailable components (Marco et al., 2017). Seaweeds (mainly kelp) are a common vegetable ingredient in the fermented food, Kimchi. The microbial content of kimchi provides a source of probiotics, nutrients, and bioactive metabolites, which are reported to have anti-microbial, anti-oxidant, and anti-obesogenic activities (Chilton et al., 2015; Tamang et al., 2016; Wilburn and Ryan, 2017). One randomised controlled trial (RCT) observed that consumption of a seaweed Kimchi made from *L. japonica* for four weeks promoted the growth

and survival of gut microbial lactic acid bacteria in humans (Seok-Jae et al., 2014), whilst another RCT concluded that consumption of 1.5 g/day fermented *L. japonica* containing 5.56% γ-aminobutyric acid (GABA) (*Lactobacillus brevis* BJ2 culture) was associated with a reduction in oxidative stress in healthy adults over four weeks, indicated by decreased serum γ-glutamyltransferse (GGT) and malondialdehyde, and increased antioxidant activity of superoxide dismutase and catalase compared to the placebo (Kang et al., 2012). The latter study indicates that foods containing fermented brown seaweeds, such as *L. japonica*, may offer a novel source of GABA enriched ingredients, which are associated with hypotensive and anti-inflammatory effects (Wilburn and Ryan, 2017). Anti-oxidant, anti-diabetic, and anti-hypertensive efficacies are also reported for Korean rice wine fermented with *L. japonica* (Choi et al., 2014), while *Sargassum* fermented with a starter culture of *Enterococcus faecium* was reported to contain higher soluble polyphenol and mannuronic acid-rich alginate contents (Shobharani et al., 2014), which may increase the provision of microbiota accessible components for colonic fermentation.

Reports of the functional properties of fermented foods containing red seaweeds are scarce; however, examples of red seaweed fermented foods include a fermented *Porphyra yezoensis* seaweed sauce, which used the marine halophilic lactic acid bacteria, *Tetragenococcus halophilus*, as a starter culture (Uchida et al., 2014); a *Gracilaria domingensis* aqueous extract applied as a texture modifier in fermented milks as a non-animal alternative to gelatin (Tavares Estevam et al., 2016); and carrageenan as a salt replacer in the production of fat-free cheese (Blaszak et al., 2018).

Given the availability of red, brown, and green seaweeds both commercially and locally (Bixler and Porse, 2011), the production of seaweed-containing fermented foods could be a cost-effective alternative to bioactive component extraction. Nevertheless, an understanding of how live bacteria and bacterial metabolites present in fermented foods contribute towards health is required (Marco et al., 2017).

3.7 Seaweeds and animal health

Seaweeds also have a historical use as animal feed ingredients (Makkar et al., 2016). The capacity for seaweeds to modulate the gut microbiota of monogastrics, such as pigs and hens, is presented in **Table 3-8 Error! Reference source not found.**and **Table 3-9**, respectively, which complements the recent evidence for the application of seaweed bioactives in monogastric animal feed (Øverland et al., 2019). **Table 3-8** shows limited evidence that the β-glucan, laminarin, may increase *Lactobacillus* populations but not *Bifidobacterium* populations. While there is scarce evidence for the selective stimulation of health-associated bacteria in pigs by the sulphated fucose, fucoidan. Only one recent study has evaluated the effect of dietary alginate on the porcine microbiota, where the genera *Ruminococcus*, *Roseburia* and *Lachnospira*, and an unclassified bacterium of the *F16* family were increased, alongside a significant decrease in the genus *Blautia,* the family *Clostridiaceae*, and an unclassified bacterium of *RF39* family (Umu et al., 2015). In **Table 3-9**, recent evidence indicates that hens fed *Chondrus crispus* and *Sarcodiotheca gaudichaudii* red seaweeds may increase ceacal SCFA concentrations and modulate populations of *Bifidobacterium longum*, *Lactobacillus acidophilus*,

Streptococcus salivarius, and *Clostridium perfringens* (Kulshreshtha et al., 2017, 2014); however, a bidirectional change in microbial composition was dose dependent and has only been assessed in two studies to date. Given the use of pigs as an animal model of humans (Litten-Brown et al., 2010), data from *in vivo* monogastric studies which are designed to evaluate the prebiotic potential of dietary seaweeds and seaweed-derived components could provide insight into the potential for human applications.

Table 3-10 and **Table 3-11** summarise recent studies which have examined the impact of seaweed diets on the ruminant microbiota of cows and sheep, respectively, with the potential application of reducing methane production. Despite demonstrating decreased methane production, the cow rumen *in vitro* fermentation studies presented in **Table 3-10** did not assess microbiota compositional changes, thus a knowledge gap is presented to understand which bacteria (if any), are increased or decreased, and are associated with a reduction in methane production. **Table 3-11** shows that methanogenic bacteria and methane production were significantly decreased compared to the basal grass substrate control following the *in vitro* fermentation of sheep rumen with the red seaweed *Asparagopsis taxiformis* (Machado et al., 2018). While sheep given an ad libitum diet of *Ascophyllum nodosum* brown seaweed (1%, 3%, or 5% *w*/*w*) for 21 days demonstrated a dose-dependent decrease in propionate and butyrate SCFAs and a dose-dependent increase in acetate (Zhou et al., 2018), while several bacteria were significantly decreased, including *Prevotella copri*, *Roseburia,* and *Coprococcus*, while *Blautia producta* and the family *Veillonellaceae* were significantly increased compared to the basal diet. Moreover, the specific case of seaweed-fed North

Ronaldsay sheep highlights how isolated organisms of the ruminant microbiome, such as *Prevotella*, *Clostridium butyricum*, *Butyrivibrio fibrisolvens*, and Spirochaetes have adapted to hydrolyse alginate laminarin, and fucoidan (Orpin et al., 1985; Williams et al., 2013). However, there is a paucity of evidence to implicate any health benefits attributed to a seaweed diet in these animals.

3.8 Conclusions

Current evidence regarding the prebiotic effects of seaweeds is dominated by complex polysaccharide components. This is because prebiotic research was previously focused on saccharolytic fermentation by the gut microbiota. Accumulating evidence from *in vitro* and *in vivo* animal studies provides encouraging data regarding the utilisation of red seaweed galactans and brown seaweed glycans, such as alginates and laminarins, with minor evidence for fucoidan and the green seaweed polysaccharide, ulvan.

Given that the most recent definition of prebiotic places non-complex polysaccharide components in vogue, an opportunity is presented to explore how other seaweed phytochemicals, including polyphenols, carotenoids, and PUFAs, are metabolised by host microbial populations to benefit host health. Future investigations should consider the use of *in vitro* screening studies and *in vivo* animal studies to identify putative prebiotic compounds from seaweeds via the identification of host organisms which utilise seaweed components and the bioactive metabolites produced (via untargeted metabolomics). Furthermore, controlled human intervention studies with health-related end points to elucidate prebiotic efficacy are required.

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3.10 Tables

Table 3-1. Potential degradation of brown seaweed glycans by the human gut microbiota.

Carbohydrate		Carbohydrate-Active Enzyme (CAZyme)	Evidenced Glycolytic Bacteria	Reference
Alginate	1,4-β-d-mannuronic acid α-l-guluronic acid	PL6 Alginate lyase PL6 MG-specific alginate lyase	*Bacteroides clarus* *Bacteroides eggerthii*	(Bai et al., 2017; Brownlee et al., 2005; Li et al., 2017; Mathieu et al., 2018; Thomas et al., 2012)
		PL15 Alginate lyase PL15 Oligoalginate lyase	*Bacteroides ovatus* *Bacteroides thetaiotaomicron* *Bacteroides xylanisolvens*	
		PL17 Alginate lyase PL17 Oligoalginate lyase	*Bacteroides clarus* *Bacteroides eggerthii*	
Fucoidan	Sulphated 1,2-1,3-1,4-α-l-fucose	GH29 α-l-fucosidase GH29 α-1,3/1,4-l-fucosidase GH95 α-l-fucosidase GH95 α-1,2-l-fucosidase	Not determined	(Zhang et al., 2009)
Laminarin	1,3-1,6-β-glucose	GH16 β-glucanase GH16 β-1,3-1,4-glucanase GH16 endo-1,3-β-glucanase	*Bacteroides distasonis* *Bacteroides fragilis* *Bacteroides thetaiotaomicron*	(Devillé et al., 2004; Salyers et al., 1977)

PL, Polysaccharide Lyase family; GH, Glycoside Hydrolase family. Potential glycolytic bacteria were identified using the Carbohydrate-Active enZYmes Database (Cantarel et al., 2008).

Table 3-2. Potential degradation of red seaweed glycans by the human gut microbiota.

Carbohydrate		Carbohydrate-Active Enzyme (CAZyme)	Evidenced Glycolytic Bacteria	Reference
Agar (Galactan)	1,3-β-d-galactose 1,4-3,6-anhydro-α-l-galactose	GH2 β-galactosidase GH16 β-agarase GH86 β-agarase GH117 1,3-α-3,6-anhydro-l-galactosidase	*Bacteroidetes plebeius*	(Dousip et al., 2014; Hehemann et al., 2010; Lahaye and Rochas, 1991; Rebuffet et al., 2011)
Carrageenan (Galactan)	1,4-β-d-galactose 1,3-α-d-galactose 3,6-anhydro-d-galactose	GH2 β-galactosidase GH117 1,3-α-3,6-anhydro-l-galactosidase	*Bacteroides plebeius*	(Hehemann et al., 2010; Weiner, 2014)
Porphyran (Galactan)	Sulphated 1,3-β-d-galactose 1,4-α-l-galactose-6-sulfate 3,6-anhydro-α-l-galactose	GH16 β-porphyranase GH86 β-porphyranase	*Bacteroides plebeius*	(Hehemann et al., 2012, 2010; Zhang et al., 2005)
Xylan	1,3-1,4-β-d-xylose	GH3 xylan 1,4-β-xylosidase GH5 endo-1,4-β-xylanase GH10 endo-1,4-β-xylanase GH10 endo-1,3-β-xylanase GH11 endo-β-1,4-xylanase GH11 endo-β-1,3-xylanase GH43 β-xylosidase GH43 xylanase GH43 β-1,3-xylosidase GH67 xylan α-1,2-glucuronidase GH115 xylan α-1,2-glucuronidase CE1–CE7 and CE12 acetyl xylanesterases	Not determined	(Despres et al., 2016; Hong et al., 2014; Mirande et al., 2010; Usov, 2011)

PL, Polysaccharide Lyase family; GH, Glycoside Hydrolase family. Potential glycolytic bacteria were identified using the Carbohydrate-Active enZYmes Database (Cantarel et al., 2008).

Table 3-3. Potential degradation of green seaweed glycans by the human gut microbiota.

Carbohydrate		Carbohydrate-Active Enzyme (CAZyme)	Evidenced Glycolytic Bacteria	Reference
Ulvan	Sulphated 1,4-β-d-Glucuronic acid	GH78 α-l-rhamnosidase	Not determined	(Lahaye and Robic, 2007; Munoz-Munoz et al., 2017)
	α-l-Rhamnose			
	1,4-β-d-xyloglucan	GH145 α-l-rhamnosidase		
Xylan	1,3-β-d-xylose	GH10 endo-1,3-β-xylanase,	Not determined	(Liang et al., 2015)
		GH11 endo-β-1,3-xylanase		
		GH43 β-1,3-xylosidase		

PL, Polysaccharide Lyase family; GH, Glycoside Hydrolase family. Potential glycolytic bacteria were identified using the Carbohydrate-Active enZYmes Database (Cantarel et al., 2008).

Table 3-4. *In vitro* fermentation of brown seaweeds with human faecal inoculum.

Seaweed	Substrate	Dose	Use of a Simulated *in vitro* Digestion Before Fermentation?	Experimental Parameters	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes	Reference
Ecklonia radiata	Crude fraction (CF) Phlorotannin-enriched fraction (PF) Low-molecular weight polysaccharide fraction (LPF) High-molecular weight polysaccharide fraction (HPF)	1.5% (*w*/*v*)	Yes CF = 71.5% digestible PF = 87.3% digestible LPF = 86.1% digestible HPF = non-digestible	10% (*w*/*v*) pooled inoculum (n = 3) 24 h	qPCR	↑ *Bifidobacterium* ↑ *Lactobacillus* (LPF) ↑ *F. prausnitzii* ↑ *C. coccoides* ↑ *Firmicutes* (CF, LPF) ↑ *Bacteroidetes* ↑*E. coli* (CF, PF, LPF, HPF) ↓ *Enterococcus* (CF, PF)	GC-FID	↑Acetate (CF) ↑Propionate (CF, LPF, HPF) ↑ Butyrate (CF, LPF, HPF) ↑ Total SCFA (CF, LPF, HPF)	(Charoensiddhi et al., 2017c)
Ecklonia radiata	Water extract (WE) Acid extract (AE) Celluclast enzyme extract (CEE) Alcalase enzyme extract (AEE) Free sugar fraction (FF) Polysaccharide fraction (PF) Seaweed residue (SR) Seaweed powder (SP)	1.5% (*w*/*v*)	No – digestibility unknown	10% (*w*/*v*) pooled inoculum (n = 3) 24 h	qPCR	= *F. prausnitzii* = *C. leptum* = *R. bromii* ↑ Total bacteria (CEE, AEE, WE, FF) ↑ *Bifidobacterium* ↑ *Bacteroidetes* ↑ *Lactobacillus* ↑ *C. coccoides* (CEE) ↑ *E. coli* ↑ *Enterocccus* (WE, AE, CEE, AEE, FF, PF, SP)	GC-FID	↑ Acetate ↑ Propionate ↑ Butyrate (WE, AE, CEE, AEE, FF, PF, SP) ↑ Total SCFA	(Charoensiddhi et al., 2016)

qPCR, Quantitative PCR; GC-FID, Gas Chromatography with Flame Ionisation Detector; SCFA, Short Chain Fatty Acid; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-4 continued. *In vitro* fermentation of brown seaweeds with human faecal inoculum.

Seaweed	Substrate	Dose	Use of a Simulated *in vitro* Digestion Before Fermentation?	Experimental Parameters	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes	Reference
Sargassum muticum	*Sargassum muticum* Alcalase enzyme extract (SAE)	1% (*w*/*v*)	Yes – non-digestible (% digestible undisclosed)	10% (*w*/*v*) single inoculum 24 h	FISH	= *Bifidobacterium* = *Lactobacillus* = *Clostridium histolticum* ↑ *Bacteroides/Prevotella* ↓ *C.coccoides/E.rectale*	HPLC	↑ Total SCFA	(Rodrigues et al., 2016)
Sargassum thunbergii	Polysaccharide extract	0.3% (*w*/*v*)	No – digestibility unknown	20% (*w*/*v*) pooled inoculum (n=3) 24 h	16S rRNA NGS	↑ Bacteroidetes ↑Bacteroidetes:Firmicutes ratio ↑ *Bifidobacterium* ↑ *Roseburia* ↑ *Parasutterella* ↑ *Fusicatenibacter* ↑ *Coprococcus* ↑ *Fecalibacterium*	GC-MS	↑ Acetate ↑ Propionate ↑ Butyrate ↑ Valerate ↑ Total SCFA	(Fu et al., 2018)

FISH, Flourescence in situ Hybridisation; 16S rRNA NGS, 16S rRNA Next Generation Sequencing; HPLC, High Performance Liquid Chromaography; GC-MS, Gas Chromatography-Mass Spectrometry; SCFA, Short Chain Fatty Acid; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-4 continued. *In vitro* fermentation of brown seaweeds with human faecal inoculum.

Seaweed	Substrate	Dose	Use of a Simulated *in vitro* Digestion Before Fermentation?	Experimental Parameters	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes
-	Alginate	5% (*w*/*v*)	No – digestibility unknown	10% (*w*/*v*) single inoculum 72 h	16S rRNA DGGE 16S rRNA NGS	↑ *Bacteroides*	GC-FID	↑ Propionate ↑ Butyrate ↑ Total SCFA
-	Alginate (A) Mannuronic acid oligosaccharides (MO) Guluronic acid oligosaccharides (GO) Propylene glycol alginate sodium sulphate (PSS)	5g/L (A) 8g/L (MO, GO, PSS)	No – digestibility unknown	10% (*w*/*v*) single inoculum 48 h	16S rRNA DGGE	Detection of *Bacteroides xylanisolvens, Clostridium clostridioforme/ Clostridium symbiosum, Bacteroides finegoldii, Shigella flexneri/E.coli, E.fergusonii,* and *Bacteroides ovatus*	HPLC	A, MO, GO: ↑ Acetate ↑ Propionate ↑ Butyrate ↑ Total SCFA
Ascophyllum nodosum	Sulphated polysaccharide extract	9 mg/mL	Yes – non-digestible (% digestible undisclosed)	10% (*w*/*v*) pooled inoculum (n = 4) 24 h	16S rRNA NGS	↑ *Bacteroides* ↑ *Phascolarctobacterium* ↑ *Oscillospira* ↑ *Fecalibacterium*	GC-FID	↑ Acetate ↑ Propionate ↑ Butyrate ↑ Total SCFA

16S rRNA DGGE, 16S rRNA Denaturing Gradient Gel Electrophoresis; 16S rRNA NGS, 16S rRNA Next Generation Sequencing; GC-FID, Gas Chromatography with Flame Ionisation Detector; HPLC, High Performance Liquid Chromaography; SCFA, Short Chain Fatty Acid; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-4 continued. *In vitro* fermentation of brown seaweeds with human faecal inoculum.

Seaweed	Substrate	Dose	Use of a Simulated *in vitro* Digestion Before Fermentation ?	Experimental Parameters	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes	Reference
Laminaria digitata	Crude polysaccharide extract (CE) Depolymerised crude polysaccharide extract (DE)	1% (*w*/*v*)	Yes – non-digestible (% digestible undisclosed)	20% (*w*/*v*) pooled inoculum (n = 3) 48 h	16S rRNA NGS	↑ *Parabacteroides* (CE, DE) ↑ *Fibrobacter* (CE) ↓ *Streptococcus* ↓ *Ruminococcus* ↑ *Lachnospiraceae* UC (DE) ↓ *Peptostreptococcaceae* IS (DE) ↑ *Dialister* (CE, DE) ↑ γ B38UC (CE)	GC-FID	↑ Acetate (CE, DE) ↑ Propionate (CE, DE) ↑ Butyrate (CE, DE) ↑ Total SCFA (CE, DE)	(Strain et al., 2019)
-	Laminarin	1% (*w*/*v*)	No – digestibility unknown	10% (*w*/*v*) pooled inoculum (n = 5) 24 h	qPCR	↑ *Bifidobacterium* ↑ *Bacteroides*	HPLC	↑ Acetate ↑ Propionate ↑ Total SCFA	(Seong et al., 2019)

16S rRNA NGS, 16S rRNA Next Generation Sequencing; qPCR, Quantitative PCR; GC-FID, Gas Chromatography with Flame Ionisation Detector; HPLC, High Performance Liquid Chromatography; SCFA, Short Chain Fatty Acid; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-5. *In vitro* fermentation of red seaweeds with human faecal inoculum.

Seaweed	Substrate	Dose	Use of a Simulated *in vitro* Digestion Before Fermentation?	Experimental Parameters	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes	Reference
Kappaphycus alvarezii	Whole Seaweed (WS)	1% (*w*/*v*)	Yes – non-digestible (% digestible undisclosed)	10% (*w*/*v*) single inoculum 24 h	FISH	↑ *Bifidobacterium* ↓ *Clostridium coccoides/ Eubacterium rectale*	HPLC	↑ Total SCFA	(Bajury et al., 2017)
Osmundea pinnatifida	*Osmundea pinnatifida* Viscozyme extract (OVE)	1% (*w*/*v*)	Yes – non-digestible (% digestible undisclosed)	10% (*w*/*v*) single inoculum 24 h	FISH	= *Bifidobacterium* = *Lactobacillus* = *Clostridium histolticum*	HPLC	↑Total SCFA	(Rodrigues et al., 2016)
Gracilaria rubra	Polysaccharide extract (PE)	1% (*w*/*v*)	Yes – non-digestible (% digestible undisclosed)	10% (*w*/*v*) pooled inoculum (n = 4) 24 h	16S rRNA NGS	↑ *Bacteroides* ↑*Prevotella* ↑*Phascolarctobacterium* ↓Firmicutes:Bacteroidetes	GC-FID	↑ Acetate ↑ Propionate ↑ Isobutyrate ↑ Total SCFA	(Di et al., 2018)
-	Porphyran	1% (*w*/*v*)	No – digestibility unknown	10% (*w*/*v*) pooled inoculum (n = 5) 24 h	qPCR	↑ *Bifidobacterium* ↑ *Bacteroides*	HPLC	= Acetate = Propionate = Butyrate = Total SCFA	(Seong et al., 2019)

FISH, Flourescence in situ Hybridisation; 16S rRNA NGS, 16S rRNA Next Generation Sequencing; qPCR, Quantitative PCR; GC-FID, Gas Chromatography with Flame Ionisation Detector; HPLC, High Performance Liquid Chromatography; SCFA, Short Chain Fatty Acid; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-6. *In vitro* fermentation of green seaweeds with human faecal inoculum.

Seaweed	Substrate	Dose	Use of a Simulated *in vitro* Digestion Before Fermentation?	Experimental Parameters	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes	Reference
Enteromorpha prolifera	Polysaccharide extract (PE)	0.2 g in 9.5 mL 0.8 g in 9.5mL	Yes-non-digestible (% digestible undisclosed)	10.5% (*w*/*v*) pooled inoculum (n = 3) 12, 24, and 48 h	Microbial culture	↑ *Enterobacter* (0.2 PE and 0.8 PE at 24 h and 48 h) = *Enterococcus* = *Lactobacillus* = *Bifidobacterium*	GC-FID	= Acetate = Butyrate = Lactate	(Kong et al., 2016)
-	Ulvan	1% (*w*/*v*)	No-digestibility unknown	10% (*w*/*v*) pooled inoculum (n = 5) 24 h	qPCR	↑ *Bifidobacterium* ↑ *Lactobacillus*	HPLC	↑ Acetate ↑ Lactate	(Seong et al., 2019)

qPCR, Quantitative PCR; GC-FID, Gas Chromatography with Flame Ionisation Detector; HPLC, High Performance Liquid Chromatography; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-7. Impact of seaweeds on the rodent gut microbiota.

Animal	Substrate	Dose	Duration	Biological Sample	Microbial Changes	Metabolite Changes	Reference
30 Male Sprague-Dawley Rats	*Chondrus crispus* Whole Seaweed (WS)	0.5% (*w*/*w*) 2.5% (*w*/*w*)	21 days	Faeces	↑*Bifidobacterium* ↑ *Legionella* ↑ *Sutterella* ↑ *Blautia* ↑ *Holdemania* ↑ *Shewanella* ↑ *Agarivorans* ↓ *Streptococcus* ↑ *Bifidobacterium breve* (2.5% WS)	↑ Acetate ↑ Propionate (2.5% WS) ↑ Butyrate ↑ Total SCFA	(Liu et al., 2015)
24 Male Sprague-Dawley Rats	*Ecklonia radiata* Whole Seaweed (WS) *Ecklonia radiata* Polysaccharide Fraction (PF)	5% (*w*/*w*) WS 5% (*w*/*w*) PF	7 days	Caecum	↑ *F. prausnitzii* ↑ *E. coli* (PF) ↓ *Enterococcus* (WS) ↓ *Lactobacillus* ↓ *Bifidobacterium* ↓ Firmicutes:Bacteroidetes	↑ Acetate ↑ Propionate ↑ Butyrate (PF) ↓ Valerate ↓ Hexanoate ↑ Total SCFA ↓ i-Butyrate ↓ i-Valerate ↓ phenol ↓ p-cresol	(Charoensiddhi et al., 2017b)
18 Male Wistar Rats	Alginate (A) Laminarin (L) Fucoidan (F)	2% (*w*/*w*)	14 days	Caecum	↑ *Bacteroides* (*Bacteroides capillosus*) Presence of *Enterorhabdus* (A) ↑ Proteobacteria. Presence of *Lachnospiracea, Parabacteroides* (*Parabacteroides distasonis*) and *Parasutterella* (L) Not fermented (F)	↑ Propionate (L) ↑ Total SCFA (A, L)	(C. An et al., 2013)

SCFA, Short Chain Fatty Acid; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-8. Impact of seaweeds on the porcine gut microbiota.

Animal	Seaweed Component	Dose	Duration	Biological Sample	Microbial Changes	Metabolite Changes	Reference
20 pregnant gilts and 48 piglets	Laminarin/Fucoidan Extract	10 g/day	Gestation (day 83) to weaning (day 28)	Faeces (Sow) Colonic digesta (Piglet)	Sows (parturition): ↓ *Enterobacteriaceae* = lactobacilli Piglets (birth, 48h after birth, weaning): = *Enterobacteriaceae* = lactobacilli	-	(Heim et al., 2015)
200 pigs	*Ecklonia cava* Whole Seaweed	0.05% (*w*/*w*) 0.1% (*w*/*w*) 0.15% (*w*/*w*)	28 days	Caecum	↑ *Lactobacillus* ↓ *E. coli* = Total Anaerobes	-	(Choi et al., 2017)
24 pigs	Laminarin/Fucoidan Extract (SD) Laminarin/Fucoidan Wet Seaweed (WS)	5.37 Kg/tonne SD 26.3 Kg/tonne WS	21 days	Ileum Caecum Colon	= *Bifidobacterium* = *Lactobacillus* = *Enterobacterium* (SD, WS) ↑ *Lactobacillus agilis* (colon)	-	(Murphy et al., 2013)
48 pigs	Laminarin Extract	300 ppm	32 days	Faeces	↑ *Lactobacillus* = Bifidobacteria	= Acetate ↓ Propionate = Butyrate = Valerate = i-Butyrate = i-Valerate	(Heim et al., 2014)
48 pigs	β-glucan	250 g/tonne 150 g/tonne	29 days	Ileum Caecum Proximal Colon Distal Colon	= lactobacilli = bifidobacteria ↑ *Lactobacillus* diversity	-	(Murphy et al., 2013)

=, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-8 continued. Impact of seaweeds on the porcine gut microbiota.

Animal	Seaweed Component	Dose	Duration	Biological Sample	Microbial Changes	Metabolite Changes	Reference
168 pigs	Laminarin (L) Fucoidan (F)	240 mg/kg F 150 mg/kg L 300 mg/kg L 150 mg/kg L and 240 mg/kg F 300 mg/kg L and 240 mg/kg F	35 days	Faeces	= *E. coli* = *Bifidobacterium* ↑ lactobacilli	= Acetate = Propionate = Butyrate = Valerate = i-Butyrate = i-Valerate = Total SCFA	(Walsh et al., 2013)
9 pigs	Alginate	5.14% (*w*/*w*)	84 days	Faeces	= Diversity ↑ Unclassified F16 family ↓ *Clostridiaceae* ↓ Unclassified RF39 (Mollicutes) ↑ *Ruminococcus* ↑ *Roseburia* ↑ unclassified *F16* genus (TM7) ↑ *Lachnospira* ↓ *Blautia*	-	(Umu et al., 2015)

=, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-9. Impact of seaweeds on the hen gut microbiota.

Animal	Seaweed Component	Dose	Duration	Biological Sample	Microbial Changes	Metabolite Changes	Reference
160 laying hens	*Chondrus crispus* Whole Seaweed (CC) *Sarcodiotheca gaudichaudii* Whole Seaweed (SG)	0.5% (*w*/*w*) 1% (*w*/*w*) 2% (*w*/*w*)	30 days	Ileum Caecal digesta	↑ *Bifidobacterium longum* (CC2, SG1, SG2) ↑ *Streptococcus salivarius* (CC1, CC2, SG2) ↓ *Clostridium perfringens* (CC1, CC2, SG1, SG2) ↓ *Lactobacillus acidophilus* (CC1, CC2)	↑ Acetate (CC1, SG1) ↑ Propionate (CC2) ↑ Butyrate (SC2)	(Kulshreshtha et al., 2014)
96 laying hens	*Chondrus crispus* Whole Seaweed (CC) *Sarcodiotheca gaudichaudii* Whole Seaweed (SG)	Control diet + 2% (*w*/*w*) seaweed Control diet + 4% (*w*/*w*) seaweed	28 days	Caecum	↑ *Lactobacillus acidophilus* (CC4) ↓ *Bifidobacterium longum* (SG2, SG4, CC4) ↓ *Streptococcus salivarius* (SG2, SG4, CC2, CC4) ↑ Bacteroidetes (SG4, CC2, CC4)	↑ Propionate (CC4)	(Kulshreshtha et al., 2017)

=, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-10. *In vitro* fermentation of seaweeds with cow rumen inoculum.

Seaweed	Substrate	Experimental Parameters	Dose (*w*/*v*)	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes	Reference
Ascophyllum nodosum (AN) *Laminaria digitata* (LD)	Whole Seaweed	50% pooled inoculum (n = 4) 24 h	0.5g/L 1g/L 2g/L	-	-	GC-FID	↑Propionate ↑Butyrate (LD) ↓ BCFA ↓Methane	(Belanche et al., 2016)
Asparagopsis taxiformis	Whole Seaweed	20% pooled inoculum (n = 4) 72 h	0.5% 1% 2% 5% 10%	-	-	GC-FID	↓ Total gas production ↓ Methane ↓ Acetate ↑ Propionate ↑ Butyrate (2%, 10%) ↓ Total SCFA (5%, 10%)	(Kinley et al., 2016)
Ulva sp. *Laminaria ochroleuca* *Saccharina latissima* *Gigartina sp.* *Gracilaria vermiculophylla*	Whole Seaweed	20% pooled inoculum (n = 2) 24 h	25%	-	-	GC-FID	↓ Methane	(Maia et al., 2016)
Brown seaweed by-products (BSB)	-	50% (*v*/*v*) single inoculum 0, 3, 6, 9, 12, and 24 h	2% 4%	-	-	GC-FID	↓ Ammonia (3, 9, 12 and 24h) ↓ Total SCFA (24h)	(Hong et al., 2015)

GC-FID, Gas Chromatography; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Table 3-11. Impact of seaweeds on the sheep rumen microbiota.

Seaweed	Dose	Experimental Parameters	Microbial Enumeration	Microbial Changes	Metabolomics Analysis Technique	Metabolite Changes	Reference
Asparagopsis taxiformis Whole Seaweed	2%	*in vitro* batch culture fermentation 20% (*v*/*v*) pooled sheep rumen fluid inoculum (n = 4) 48 and 72 h	16S rRNA NGS qPCR	↓Methanogens ↓Bacteroidetes/Firmicutes ratio ↓ mcrA gene expression	GC-MS	↓ Total Gas ↓ Methane ↑ Hydrogen	(Machado et al., 2018)
Ascophyllum nodosum Whole Seaweed	1% 3% 5%	Rams (n = 8) 21 days *ad libitum*	16S rRNA NGS	↓ *undefined TM7-1* ↓*undefined Coriobacteriaceae* ↓*Roseburia* ↓*Coprococcus* ↓*Prevotella copri* ↑*Blautia producta* ↑ *Entodinium species 1* ↑*Veillonellaceae*	GC-FID	*Dose dependent:* ↑ *Acetate* ↓ *Propionate* ↓ *Butyrate* PICRUSt: ↑Butanoate metabolism ↑ Fatty acid metabolism ↓Glycerophospholipid metabolism	(Zhou et al., 2018)

16S rRNA NGS, 16S rRNA Next Generation Sequencing; qPCR, Quantitative PCR; GC-FID, Gas Chromatography; =, no statistical difference compared to the control; ↑, significant increase compared to the control; ↓ significant decrease compared to the control. Microbial and metabolite changes with abbreviations in parenthesis indicate the substrate(s) which exerted the effect. If no abbreviations in parenthesis are presented, then all of the seaweed substrates tested exerted the effect.

Chapter 4

Experimental Chapter

4 Effects of the edible Irish brown seaweeds, *Fucus vesiculosus* and *Laminaria digitata*, on the composition and metabolic activity of the human gut microbiota using an *in vitro* model of the distal colon.

4.1 Abstract

There is increasing interest in the health benefits of brown seaweeds, with many postulating that the fibre component may confer health benefits due to its prebiotic properties. This study investigated the prebiotic potential of *Fucus vesiculosus* and *Laminaria digitata* whole seaweed powders (FVWS and LDWS, respectively), as well as their isolated polysaccharide rich extracts (FVE and LDE, respectively), using *in vitro* faecal batch culture fermentation experiments. The effect of the seaweed treatments on microbiota composition (qPCR & 16S rRNA amplicon sequencing) and metabolism (short chain fatty acids via GC-MS) was compared with cellulose (negative control) and Synergy 1 (positive control).

None of the seaweed substrates stimulated the growth of *Bifidobacterium* spp. when compared to cellulose (qPCR), whilst significant reductions of *Lactobacillus* spp. were evident in LDWS (5 & 10 hrs) and LDE (5 hrs). 16S rRNA amplicon sequencing showed no effect of seaweed treatments on alpha and beta measures of bacterial diversity when compared to cellulose. Inclusion of FVE in the fermentation showed a stimulation of the genera *Phascolarctobacterium*, *Oscillospira*, and *Faecalibacterium*, and several genera within the *Lachnospiraceae*, *Prevotellaceae*, and *Ruminococcaceae* families when compared to cellulose. LDWS treatment showed significant changes in the genera *Bacteroides*, *Coprococcus*, and *Parasutterella*, in addition to genera from the families *Ruminococcaceae* and

Lachnospiraceae when compared to cellulose. LDE treatment showed significant changes in the genera *Butyricimonas*, Coprococcus, *Phascolarctobacterium, Pseudobutyrivibrio* and *Roseburia* when compared to cellulose. FVWS and FVE treatment resulted in significantly higher concentrations of acetic acid (24, 36 & 48 h, $p < 0.05$) when compared to cellulose. FVWS treatment also resulted in significantly higher propionic acid concentrations when compared to cellulose after 48 h. LDWS treatment resulted in significantly higher concentrations of acetic acid (10 h, $p < 0.05$) and butyric acid (24 & 36 h, $p < 0.05$) and total SCFA (24 & 36 h, $p < 0.05$) when compared to cellulose. This study showed that neither seaweed mediated bifidogenic effects nor altered bacterial diversity, however, the noted increases in short chain fatty acid concentrations, particularly following the whole seaweed treatments, warrant further research in an *in vivo* setting.

4.2 Introduction

Beneficial effects of adequate dietary fibre intake are associated with a reduced risk and mortality of a range of non-communicable diseases (NCDs) such as obesity, type 2 diabetes, and cardiovascular disease (Reynolds et al., 2019). The recommended fibre intake can vary greatly between 25-38 g/day, depending on the country; however, many populations struggle to achieve adequate intakes, and this is suggested to negatively impact health (EFSA, 2010; US Institute of Medicine, 2001). The health benefits of fibre, beyond the established faecal bulking properties, have been attributed to its viscosity and gel-forming properties, which can influence gastrointestinal transit time to impact appetite, nutrient absorption and metabolism (Mattea Müller et al., 2018). Additionally, high fibre diets have been shown to promote increased gut bacterial diversity, and increase the

abundance and metabolic activity of beneficial microorganisms (O'Grady et al., 2019).

The fermentation of dietary fibre components in the lower GI tract, dictated by the expression of enzymes present in the human gut microbiome, has been shown to modulate changes in gut microbiota composition, diversity, and metabolic output (O'Grady et al., 2019). Such modulation may exert positive effects on the intestinal epithelium, immune cells, and systemically, beyond the gut where substrates (including dietary fibre), that are selectively utilized by host microorganisms to confer a health benefit, are considered prebiotics (Gibson et al., 2017).

The most researched commercially available prebiotics, inulin, fructo-oligosaccharides and galacto-oligosaccharides have historically focused on the selective enhancement of *Bifidobacterium* and *Lactobacillus* and the production of short chain fatty acids (SCFA), as candidate mediators of health. SCFA are a group of fermentation metabolites which are proposed to exert beneficial effects on health by affecting host physiology via the regulation of gut barrier function, control of appetite, glucose and lipid homeostasis as well as immunomodulation (O'Grady et al., 2019; Chambers et al., 2018). The microbial compositional and metabolic changes that can be deemed as indicators of prebiotic activity continue to evolve with the development of next-generation sequencing and metabolomics analysis techniques. More recently, the accumulating evidence to implicate increased gut microbial diversity with positive health outcomes, has increased attention on microbial function and diversity as indicators of prebiotic potential.

There is increasing interest in the identification of alternative sources of prebiotic fibres, where seaweeds represent a potentially novel source of prebiotic compounds owing to the presence of non-digestible polysaccharides that are not generally found in other dietary sources. For brown seaweeds, this includes alginate, laminarin, and fucoidan polysaccharides. Previous batch culture experiments have provided indications of prebiotic activity of isolated fibre components from brown seaweeds, however, a variability in experimental parameters and analysis limits their interpretation.

This study aims to examine the prebiotic potential of Irish-sourced *Fucus vesiculosus* (*F. vesiculosus*) and *Laminaria digitata* (*L. digitata*) brown seaweeds, by investigating the fermentability of *F. vesiculosus* powder (FVWS) and *L. digitata* powder (LDWS), and polysaccharide-rich complex carbohydrate extracts of *F. vesiculosus* (FVE) and *L. digitata* (LDE), using an *in vitro* model of human colonic fermentation in comparison to cellulose and Synergy 1 negative and positive controls, respectively.

4.3 Materials and Methods

4.3.1 Chemicals and reagents

Chemicals were purchased from Sigma Aldrich (St. Louis, USA) unless otherwise stated. Reagents used during HPSEC and GC-MS were HPLC grade. Reagents used for DNA extraction, qPCR, and 16S rRNA amplicon sequencing were molecular biology grade.

4.3.2 Seaweed harvest

F. vesiculosus and *L. digitata* were harvested from Spiddal, Co. Galway, Ireland (53° 14' 48" North, 9° 18' 10" West) in September 2014. Only the leaf was used, and the

stipe was removed from the holdfast during harvesting. The seaweed was immediately washed in seawater to remove contaminants (e.g. epiphytes, molluscs and other seaweeds). Washed seaweed was freeze-dried, ground into a fine powder using an electronic blender, and stored at -20 °C until required.

4.3.3 Polysaccharide-rich extract production

F. vesiculosus and *L. digitata* powders were shaken in dH_2O (1:20, w/v) for two mins to reduce the initial salt content of the seaweed and then filtered through muslin cloth to remove the water. A total of 30g of washed seaweed was immediately freeze-dried and stored for *in vitro* digestion and the batch culture fermentation experiments (FVWS and LDWS). Seaweed powders then underwent hot acid extraction, neutralisation, desalination, ethanol precipitation, and lyophilisation to generate crude *F. vesiculosus* and *L. digitata* polysaccharide-rich extract powders (FVE and LDE, respectively).

4.3.4 FVE and LDE characterisation

The average molecular weight of FVE and LDE was determined using a modified high performance size exclusion chromatography method (Gomez-Ordonez et al., 2012). In brief, separation was performed on a PL aquagel-oh mixed-H 8µm SEC analytical column (7.5 x 300 mm i.d) with isocratic elution at 50°C and a flow rate of 0.6 mL/min and a run time of 31 mins, using a 50mM ammonium formate mobile phase and a ten-point pullulan standard curve (0.34 to 708 kDa).

Total carbohydrate content of FVE and LDE was quantified using a modified phenol-sulfuric acid colorimetric assay (DuBois et al., 1956). Sulphate content of FVE and LDE was quantified using a modified Azure A colorimetric assay, expressed as percentage equivalents of purified fucoidan (Fucoidan from *Fucus vesiculosus*

F5631, Sigma Aldrich, USA) (Torode et al., 2015). Structural information of FVE and LDE was determined using Fourier Transform Infrared Spectroscopy (FTIR) using the Bruker Tensor 27 FT-IR spectrophotometer (Bruker Corporation, UK) with OPUS 5.5 software.

4.3.5 Mineral and trace element content of FVWS, LDWS, FVE and LDE

Mineral and trace element analysis of FVWS, LDWS, FVE and LDE (1 x 10 g of each) was outsourced to Advanced Laboratories (Salt Lake City, Utah, USA). Concentrations of aluminium, copper, sodium and zinc were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Concentrations of mercury, lead, arsenic, and cadmium were determined by inductively coupled plasma mass spectrometry (ICP-MS). Iodine concentration was determined using AOAC 932.21.

4.3.6 *In vitro* digestion

An *in vitro* simulated digestion was completed on FVWS, LDWS, FVE and LDE using a standardised method (Minekus et al., 2014) with oral, gastric, and intestinal phases of digestion. All simulated fluids were incubated at 37°C before use and at all digestion stages the fluids were incubated at 37°C in an orbital shaker (160 rpm). The oral suspension was incubated for 2 mins, while both the gastric and intestinal suspensions were incubated for 2 hrs. The intestinal phase digesta was then dialysed for 24 hrs using 1kDa dialysis tubing (Spectrum Labs, USA) and the retentate was freeze dried (Labconco, USA) to obtain powders prior to batch culture fermentation.

4.3.8 *In vitro* batch culture fermentation

A 20% faecal slurry was prepared as described by O'Donnell *et al*. (O'Donnell et al., 2016). The resulting faecal bacteria suspension was amended with sterile glycerol to a final concentration of 25% (v/v) and stored frozen at -80°C until use. Nutrient basal medium stock solution was prepared using methodology of Fooks and Gibson (Fooks and Gibson, 2003). *In vitro* batch culture fermentation was performed using the Multifors parallel bioreactor with Iris 6.0 software (Infors HT, Basel, Switzerland). A total of 2g of either FVWS, LDWS, FVE, LDE, cellulose (negative control), or Synergy 1 (positive control) was added to 190ml nutrient basal medium stock solution (final carbohydrate concentration = 1% w/v). Vessels were sparged with N_2 gas for 2 hrs (2 psi) before inoculation with 10ml of 20% faecal slurry (previously thawed at 37°C). Vessels were stirred at 200 revolutions min^{-1}, maintained at pH 6.8 and 37°C. A 7mL sample was taken at t = 0, 5, 10, 24, 36 and 48 hrs for culture-dependent, culture-independent (qPCR and 16S rRNA amplicon sequencing), and SCFA analysis. Substrate fermentation was performed in triplicate.

4.3.9 DNA extraction

Genomic DNA was extracted from samples after 0, 5, 10, 24, 36, and 48 hrs fermentation using the PowerFecal DNA extraction kit (Mo Bio Laboratories, Carlsbad, USA) according to manufactures' instructions. The bead beating step was completed using the Mo Bio vortex adapter.

4.3.10 qPCR

Total bacteria, *Lactobacillus*, and *Bifidobacterium* were quantified using qPCR. The primer sequences used for qPCR were: (Target: Forward primer 5'-3'; Reverse

primer 5'-3'; Size bp; Tm °C); Total Bacteria (Eubacterial): ACTCCTACGGGAGGCAGCAG; ATTACCGCGGCTGCTGG; 200 bp; 60°C; *Lactobacillus* genus: GCAGCAGTAGGGAATCTTCCA; GCATTYCACCGCTACACATG; 349 bp; 60°C; and *Bifidobacterium* genus: CTCCTGGAAACGGGTGGT; GCTGCCTCCCGTAGGAGT; 203 bp; 60°C. A standard curve of 10^9-10^3 CFU/ml was prepared in duplicate for each plate. A PCR master mix was prepared with the forward and reverse primers, SYBR® FAST pPCR Master Mix (KAPA Biosystems, USA), and PCR water. 1 µl of sample DNA or PCR water (negative control) was added to 9 µl master mix per well (reaction volume = 10 µl) and ran in duplicate on two plates (n = 4). The Lightcycler® 480 Instrument II (Roche, Switzerland) was used with the following PCR conditions: denaturation = 1 cycle; amplification = 40 cycles; melting = 1 cycle; cooling = 1 cycle. Target temperature was 95°C with a hold time of 3 min and a ramp rate of 4.4°C/sec.

4.3.11 16S rRNA amplicon sequencing

Illumina MiSeq sequencing library preparation was completed following the 16S metagenomic sequencing library protocol (Illumina, USA) and as described by Fouhy *et al*. (Fouhy et al., 2015). Amplicon PCR: Genomic DNA was amplified using primers specific to the V3-V4 hypervariable region of the 16S ribosomal RNA gene to create a 460bp amplicon. These primers also incorporated the Illumina overhang adaptor (Forward primer 5’

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

reverse primer 5’

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Each PCR reaction contained 2.5 µL template DNA, 5 µL forward primer (1 µM), 5

µL reverse primer (1 µM), 12.5 µL 2X Kapa HiFi Hotstart ready mix (KAPA Biosystems, USA) giving a total of 25µL in the final reaction volume. PCR amplification was carried out using the Applied Biosystems 2720 thermal cycler (Life Technologies, USA) with the following parameters: heated lid 110°, 95°C for 3 mins; then 25 cycles of: 95°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs; hold at 72°C for 5 mins; hold at 4°C. Successful PCR products were cleaned using Agentcourt AMPure XP kit (Beckman Coulter Genomics, UK). A second PCR reaction was completed to attach the Illumina sequencing adapters onto the amplicons using the Nextera XT Index kit (Illumina, USA). The DNA concentration of each sample was determined using the Qubit High Sensitivity DNA kit and the Qubit 3 Fluorometer (Invitrogen, USA). DNA samples were then pooled as an equimolar mix and sequenced on the MiSeq sequencing platform at Teagasc, Moorepark, Ireland following standard Illumina sequencing protocols for the 2 × 250 cycle V3 Kit.

4.3.12 Bioinformatics

Two hundred and fifty base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (Caporaso et al., 2010). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for

aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123.

4.3.13 Short chain fatty acid and branched chain fatty acid analysis

GC-MS analysis was carried out using a modified protocol described by Garcia-Villalba *et al.* (Garcia-Villalba et al., 2012). In brief, phosphoric acid was added to samples to a final concentration of 0.5 % (v/v) prior to ethyl acetate extraction (1:1 v/v). 180 µl organic phase was added to a GC vial alongside 20 µl of 4-methyl valerate internal standard. A standard curve of 10, 20, 50, 100, 500, 1000, 5000, 10000, 50000, and 100,000 µM SCFA mix containing acetic acid, propionic acid, n-butyric acid, i-butyric acid, valeric acid, i-valeric acid, and hexanoic acid was run within every sample batch. Quality control consisted of two 50 µM and two 100 µM standard mixes every sixteen vials and ethyl acetate blanks every six vials, and between each standard vial/QC to prevent carryover. The GC-MS system consisted of an Agilent 6890N (Agilent Technologies, USA), equipped with an Agilent 7683 AutoSampler and 7683B injector, coupled to an Agilent 5973 inert mass selective detector. Agilent MassHunter GC/MS Acquisition software was used. The GC was fitted with a DB-WAXetr capillary column (30m length, 0.25mm i.d, 0.25µm film thickness), with helium used as the carrier gas (1.2mL/min). Injections were made in splitless mode with an injection volume of 1µL (10 µL syringe) and an injection temperature of 250°C. The syringe undertook four pre-washes and four post-washes in hexane. The initial column temperature was 90°C and ramped to 150°C at 15°C/min, then to 170°C at 5°C/min, then to 230°C at 20°C/min, where it was maintained for 2 mins. Total run time was 14 mins. Solvent delay was 2.5 mins. The detector was operated in electron impact ionisation mode.

4.3.14 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 24) and GraphPad Prism 8 software. Graphs were made in GraphPad Prism 8 software. All statistical tests compared each individual fermentation substrate to the cellulose negative control. For qPCR data, statistical significance was determined using a non-parametric Kruskal-Wallis test (*p* value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (*q* value) with a significance level of ≤ 0.05. For SCFA data, statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons with a significance level of $p \leq 0.05$.

Statistical analysis of 16S rRNA amplicon sequencing data was carried out using Calypso online software (version 8.68) (Zakrzewski et al., 2017). Data were normalized using cumulative sum scaling and $\log_2$ transformed to account for the non-normal distribution of sequencing data (Paulson et al., 2013). Up to 20,000 taxa with > 0.01% abundance were used in the analysis. Chloroplasts and cyanobacteria were removed from the analysis.

Alpha diversity was determined using rarefied Chao1, Evenness, Shannon and Simpson indices. Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). Beta diversity was determined Bray-Curtis dissimilarity distance matrices for each fermentation substrate and cellulose at a given time point. A Permutational multivariate analysis of variance (PerMANOVA) was used to determine the statistical difference between Bray–Curtis dissimilarity indices of beta diversity.

Statistical significance of mean bacterial relative abundances, compared to cellulose, at the phylum, family, and genus level after 0, 10, and 24 hrs fermentation was determined using a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons and a significance level of $p \leq 0.05$. Discriminate taxa between fermentation substrates and cellulose at a given time point were identified using linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011).

4.4 Results

4.4.1 FVE and LDE characterisation

The average molecular weight of FVE and LDE were 6.2 kDa and 6.1 kDa, respectively. The total carbohydrate content of FVE and LDE were 86.50 ± 5.27 % and 99.72 ± 0.79 %, respectively. While the total sulphate content of FVE and LDE were 21.41 ± 0.11 % and 9.06 ± 0.99 % fucoidan equivalents, respectively. **Figure 4-1** shows the FT-IR spectra of FVE and LDE pre- and post- *in vitro* digestion.

The spectrum of FVE showed absorption maxima indicative of the presence of alginate and/or laminarin from 1095 – 970cm^{-1} (Gomez-Ordonez et al., 2012; Yuan and Macquarrie, 2015). The band at 1625cm^{-1} indicated an uronic acid (Rioux et al., 2010), while the 1265 – 1214cm^{-1} band indicated S=O vibration of a sulphated ester which may be attributed to fucoidan (Yuan and Macquarrie, 2015). Bands at 970 – 939cm^{-1} indicate a CH_3 or C-OH bend and absorbance at 883cm^{-1} showed a sulphated glucose (C6) (Ji et al., 2011; Teodosio Melo et al., 2013). Post-digestion spectra demonstrated structural similarity with the main carbohydrate band overlapping from 1084 – 1033 cm^{-1} with an asymmetric S=O also present at 1267 –

1221 cm^{-1}, where bands between 1537 – 1221 cm^{-1} indicated C-S and O=S=O sulphate esters (Jiao et al., 2012).

The spectrum of LDE showed a large (and overlapping) band between 1083 and 931 cm^{-1} with strongest absorbance at 1022 cm^{-1} and 1083 cm^{-1}. This corresponds with a C-O and C-C pyranose ring of constituent polysaccharides such as alginate, laminarin or fucoidan (Gomez-Ordonez et al., 2012; Yuan and Macquarrie, 2015). Overlapping bands between 1265 and 1216 cm^{-1} are indicative of S=O vibration from a sulphated ester (Ji et al., 2011; Teodosio Melo et al., 2013; Yuan and Macquarrie, 2015). Two notable bands at 970 – 931 cm^{-1} and 890 – 877 cm^{-1} represent C-O-S of sulphated glucose (C6) and a CH_3 or COH bend (Ji et al., 2011; Teodosio Melo et al., 2013). Weak absorption at 1454cm^{-1} could indicate a C-O or C-H stretch; at 1344cm^{-1} a C-H stretch; and at 1133cm^{-1}, a C-O-S=O stretch. The maxima of the post-digestion extract (1030 cm^{-1}) may correspond with alginate. Prominent bands in the post-digestion extract included bands at 1641, 1419 - 1390 and 1263 – 1218 cm^{-1}, where the latter indicated an asymmetric S=O sulphated ester.

4.4.2 Mineral and trace element analysis

Table 4-1 and **Table 4-2** present data from the mineral and trace element analysis for *Fucus vesiculosus* and *Laminaria digitata* substrates, respectively. Of note, the iodine content of FVWS and FVE were 0.22 mg/g and 0.09 mg/g, respectively (**Table 4-1**). While the iodine content of LDWS and LDE were 0.163 mg/g and 0.13 mg/g, respectively (**Table 4-2**).

4.4.4 Batch culture fermentation

4.4.4.1 Effects on microbial composition as assessed by qPCR

There were no significant differences in the number of total bacteria (**Figure 4-2**) or *Bifidobacterium* spp. (

Figure 4-4) present in vessels treated with any test substrate when compared to cellulose at any time point ($q > 0.5$). Populations of *Lactobacillus* spp. were significantly lower than the cellulose control in vessels treated with LDWS after 5 hrs ($q = 0.0038$) and 10 hrs ($q = 0.0071$), and LDE after 10 hrs ($q = 0.0066$) (

Figure 4-3).

4.4.4.2 Effects on microbial composition as assessed by 16S rRNA amplicon sequencing

There were no significant differences between any test substrates when compared to cellulose for the alpha diversity indices of Chao1, Evenness, Simpson Index and Shannon Index after 10 hrs ($q > 0.5$) (**Figure 4-5**) or 24 hrs ($q > 0.1$) (**Figure 4-6**); nor for the Bray-Curtis dissimilarity index of beta diversity after 10 hrs or 24 hrs ($p > 0.5$).

There were no significant differences in the Firmicutes to Bacteroides ratio in vessels treated with any test compound when compared to cellulose at any time point. After 10 hrs fermentation, however, the Firmicutes to Bacteroidetes ratio (mean ± SD) was significantly higher in vessels treated with Synergy 1 (5.90 ± 0.37) when compared to FVWS (2.36 ± 0.33, $p = 0.0128$), FVE (2.20 ± 1.43, $p = 0.009$), and LDWS (2.13 ± 0.33, $p = 0.008$) (**Figure 4-7**)**.**

The relative abundance of the bacteria present in vessels treated with cellulose, FVWS, FVE, LDWS, LDE, and Synergy 1, are shown at the phylum level (**Figure 4-8**), family level (**Figure 4-9**), and genus level (**Figure 4-10**, **Figure 4-11**, and **Figure 4-12**), after 0, 10, and 24 hrs fermentation.

Significant differences in bacterial taxonomy at the phylum, family, and genus level, after 10 and 24 hrs fermentation with FVWS, FVE, LDWS, LDE, and Synergy 1 substrates when compared to cellulose are shown in **Table 4-3**, **Table 4-4**, **Table 4-5**, **Table 4-6**, and **Table 4-7**, respectively. The relative abundance of the genera *Bifidobacterium* and *Lactobacillus* were not significantly different in vessels treated with any of the seaweed substrates (FVWS, FVE, LDWS or LDE) at any time point when compared to cellulose.

Fucus vesiculosus whole seaweed (FVWS)

Table 4-3 shows that after 10 hrs fermentation in the presence of FVWS, the relative abundance of several genera was significantly higher when compared to cellulose. This included *Blastopirellula* (1.110 ± 0.165 % vs < 0.001 %, $p < 0.0001$), *Granulosicoccus* (0.770 ± 0.288 % vs < 0.001 %, $p < 0.0001$), and *Klebsiella* (3.837 ± 0.600 % vs 1.448 ± 0.362, $p = 0.05$). While fermentation in the presence of FVWS resulted in a significantly lower relative abundance of the genera *Haemophilus* (0.193 ± 0.168 % vs 0.885 ± 0.067 %, $p = 0.034$), *Lachnospiraceae FCS020 group* (0.081 ± 0.070 % vs 0.313 ± 0.107 %, $p = 0.033$), *Peptoclostridium* (0.773 ± 0.098 % vs 8.856 ± 0.976 %, $p < 0.0001$), *Ruminiclostridium* (0.375 ± 0.070 % vs 0.707 ± 0.100 %, $p = 0.040$), *Ruminococcus* 1 (0.291 ± 0.045 % vs 0.586 ± 0.032 %, $p = 0.012$), and *Terrisporobacter* (0.311 ± 0.052 % vs 1.505 ± 0.134 %, $p < 0.0001$) when compared to cellulose.

LEfSe analysis showed that fermentation in the presence of FVWS was associated with the genera *Blastopirellula*, *Citrobacter*, *Dorea*, *Enterobacter*, *Granulosicoccus*, *Klebsiella*, and an unclassified bacterium after 10 hrs when compared to cellulose (**Figure 4-13**).

After 24 hrs fermentation in the presence of FVWS, the relative abundance of the genus *Blastopirellula* was significantly higher in FVWS treated vessels when compared to cellulose (0.838 ± 0.419 % vs < 0.001 %, p = 0.001). While fermentation in the presence of FVWS resulted in a significantly lower relative abundance of several genera when compared to cellulose, including the *Eubacterium hallii group* (0.640 ± 0.215 % vs 0.300 ± 0.174 %, p = 0.04), *Flavonifractor* (0.691 ± 0.191 % vs 0.125 ± 0.049 %, p = 0.045), *Pseudoflavonifractor* (< 0.001 % vs 0.517 ± 0.174 %, p = 0.044), and *Ruminococcaceae UCG003* (0.139 ± 0.133 % vs 0.884 ± 0.434 %, p = 0.034) (**Table 4-3**).

LEfSe analysis showed that fermentation in the presence of FVWS was associated with the genera *Blastopirellula*, *Butyricimonas*, *Granulosicoccus*, and an unclassified bacterium after 24 hrs when compared to cellulose (**Figure 4-13.** LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) *F. vesiculosus* whole seaweed (FVWS) when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.**Figure 4-13**).

Fucus vesiculosus polysaccharide-rich extract (FVE)

After 10 hrs fermentation in the presence of FVE, the relative abundance of several genera was significantly higher when compared to cellulose. This included the genera *Alistipes* (1.704 ± 0.148 % vs 1.134 ± 0.138 %, p = 0.0389), *Christensenellaceae R7 group* (0.866 ± 0.056% vs 0.436 ± 0.018 %, p = 0.027), *Coprococcus 2* (0.746 ± 0.013 % vs 0.078 ± 0.135 %, p = 0.005), *Eubacterium coprostanoligenes group* (1.347 ± 0.558 % vs 0.119 ± 0.109 %, p = 0.002), *Eubacterium ruminantium group* (0.268 ± 0.094 % vs < 0.001 %, p = 0.001), *Eubacterium ventriosum group* (0.297 ± 0.054 % vs 0.089 ± 0.085 %, p = 0.035), *Lachnospiraceae NC2004 group* (0.389 ± 0.159 % vs 0.033 ± 0.056 %, p = 0.007), *Lachnospiraceae NK4A136 group* (0.602 ± 0.096% vs 0.218 ± 0.033 %, p = 0.014), *Lachnospiraceae UCG001* (0.322 ± 0.082 % vs < 0.001 %, p = 0.00011), *Oscillospira* (0.241 ± 0.061 % vs < 0.001 %, p = 0.021), *Prevotella 7* (1.544 ± 0.706 % vs 0.369 ± 0.143 %, p = 0.01), *Pseudobutyrivibrio* (1.654 ± 0.231 % vs 0.675 ± 0.156 %, p = 0.001), and *Ruminococcaceae UCG005* (0.766 ± 0.114 % vs 0.371 ± 0.088, p = 0.018) (**Table 4-4**). Furthermore, fermentation in the presence of FVE resulted in a significantly lower relative abundance of three genera when compared to cellulose: *Megasphaera* (2.549 ± 0.223 % vs 9.518 ± 1.665 %, p = 0.005), *Peptoclostridium* (0.570 ± 0.030 % vs 8.856 ± 0.976 %, p < 0.0001), and *Terrisporobacter* (0.443 ± 0.081 % vs 1.505 ± 0.134%, p < 0.0001) (**Table 4-4**).

LEfSe analysis showed that fermentation in the presence of FVE was associated with several genera belonging to the Eubacteriaceae, *Lachnospiraceae*, *Prevotellaceae*, and *Ruminococcaceae* families after 10 hrs when compared to cellulose (**Figure 4-14**), as well as *Alistipes, Alloprevotella*, *Anaerostipes,*

Anaerotruncus, *Coprococcus 2, Christensenellaceae R7 group*, *Dorea*, *Faecalibacterium,* the *Family XIII AD3011 group Hungatella, Incertae Sedis group, Megamonas*, *Oscillospira*, *Pseudobutyrivibrio,* and *Yersinia*.

After 24 hrs fermentation in the presence of FVE, the relative abundance of the genera *Eubacterium hallii group* was significantly higher when compared to cellulose (0.297 ± 0.057 % vs 0.640 ± 0.215 %, p = 0.038). Whereas the relative abundance of two genera were significantly lower when compared to cellulose: *Megasphaera* (5.147 ± 2.515 % vs 10.625 ± 0.288 %, p = 0.007) and *Ruminococcaceae UCG003* (0.060 ± 0.105 % vs 0.884 ± 0.434 %, p = 0.018) (**Table 4-4**).

LEfSe analysis showed that fermentation in the presence of FVE was associated with the *Christensenellaceae R7 group*, *Enterococcus*, *Erysipelotrichaceae UCG003*, *Escherichia/Shigella, Phascolarctobacterium*, *Prevotella 7*, and *Veillonella*, when compared to cellulose (**Figure 4-14**).

Laminaria digitata whole seaweed (LDWS)

Table 4-5 shows that after 10 hrs fermentation in the presence of LDWS, the relative abundance of three genera were significantly higher when compared to cellulose. This included *Coprococcus 2* (0.584 ± 0.209 % vs 0.078 ± 0.135 %, p = 0.032), *Eubacterium ventriosum group* (0.302 ± 0.077 % vs 0.089 ± 0.085%, p = 0.03), and *Lachnospiraceae NK4A136 group* (0.565 ± 0.136 % vs 0.218 ± 0.033 %, p = 0.028). Fermentation in the presence of LDWS also resulted in a significantly lower relative abundance of three genera when compared to cellulose, namely *Eubacterium hallii group* (0.090 ± 0.156 % vs 0.568 ± 0.055 %, p = 0.006),

Peptoclostridium (0.562 ± 0.318 % vs 8.856 ± 0.976 %, $p < 0.0001$), and *Terrisporobacter* (0.142 ± 0.130 % vs 1.505 ± 0.134 %, $p < 0.0001$).

LEfSe analysis showed that *Coprococcus 2*, *Escherichia/Shigella*, *Eubacterium ventriosum*, *Family XIII AD3011 group*, *Lachnospiraceae NK4A135 group*, *Lachnospiraceae UCG008,* and an uncultured bacterium were discriminative bacteria fermentation in the presence of LDWS when compared to cellulose after 10 hrs (**Figure 4-15**).

After 24 hrs fermentation in the presence of LDWS, the relative abundance of four genera were significantly higher when compared to cellulose, namely *Butyricimonas* (0.577 ± 0.005 % vs 0.234 ± 0.058%, $p < 0.001$), *Coprococcus 2* (0.382 ± 0.205 % vs 0.052 ± 0.091 %, $p = 0.018$), *Parasutterella* (0.909 ± 0.081 % vs 0.606 ± 0.069 %, $p = 0.043$), and *Ruminococcaceae UCG004* (0.056 ± 0.050 % vs < 0.001 %, $p = 0.049$) (**Table 4-5**).

Whereas fermentation in the presence of LDWS resulted in a significantly lower relative abundance of the *Eubacterium hallii group* (0.119 ± 0.018 % vs 0.640 ± 0.215 %, $p = 0.002$) and the genera *Ruminococcaceae UCG003* (0.180 ± 0.158 % vs 0.884 ± 0.434 %, $p = 0.048$) after 24 hrs when compared to cellulose (**Table 4-5**).

LEfSe analysis determined that *Bacteroides*, *Butyricimonas*, *Coprococcus 2*, *Escherichia/Shigella, Parasutterella*, *Ruminococcaceae UCG003*, *Ruminococcus 2*, and *Veillonella*, were discriminative bacteria of fermentation in the presence of LDWS when compared to cellulose (**Figure 4-15**).

Laminaria digitata polysaccharide-rich extract (LDE)

10 hrs fermentation in the presence of LDE resulted in a significantly higher relative abundance of the genera *Serratia* (7.451 ± 3.981 % vs 0.030 ± 0.051 %, $p = 0.001$) and *Yersinia* (4.127 ± 0.720 % vs 0.099 ± 0.047 %, $p < 0.0001$) when compared to cellulose. Whereas the relative abundance of the genus *Peptoclostridium* (5.639 ± 1.172 % vs 8.856 ± 0.976 %, $p = 0.002$) and *Terrisporobacter* (1.030 ± 0.188 % vs 1.505 ± 0.134 %, $p = 0.012$) were significantly lower when compared to cellulose (**Table 4-6**).

After 10 hrs, LEfSe analysis showed that fermentation in the presence of LDE was associated with the genera *Asteroleplasma*, *Catenibacterium*, *Coprococcus 2*, *Enterococcus*, *Phascolarctobacterium*, *Pseudobutyrivibrio*, *Serratia*, *Streptococcus*, *Yersinia*, and an unclassified bacterium, when compared to cellulose (**Figure 4-16**).

After 24 hrs, fermentation in the presence of LDE resulted in a significantly higher relative abundance of the genera *Solobacterium* (2.150 ± 1.343 % vs 0.182 ± 0.072 %, $p = 0.016$), *Serratia* (4.460 ± 3.525 % vs 0.081 ± 0.141 %, $p = 0.027$) and *Yersinia* (0.571 ± 0.348 % vs 0.089 ± 0.154 %, $p = 0.032$) when compared to cellulose. Whereas the relative abundance of the *Eubacterium hallii group* was significantly lower after 24 hrs fermentation in the presence of LDE when compared to cellulose (0.234 ± 0.049 % vs 0.640 ± 0.215 %, $p = 0.013$) (**Table 4-6**).

After 24 hrs, LEfSe analysis showed that fermentation in the presence of LDE was associated with the genera *Butyricimonas*, *Clostridium sensu stricto 1*, *Erysipelatoclostridium*, *Erysopelotrichaceae UCG003*, *Holdemanella*,

Lachnospiraceae UCG008, *Pseudobutyrivibrio*, *Roseburia*, *Serratia*, *Solobacterium*, and *Streptococcus* (**Figure 4-16**).

Synergy 1

Fermentation in the presence of Synergy 1 resulted in a significantly higher relative abundance of *Bifidobacterium* after 10 hrs ($p = 0.001$) and 24 hrs ($p < 0.0001$). The significant differences in bacterial composition following Synergy 1 fermentation when compared to cellulose are presented in **Table 4-7**.

After 24 hrs, LEfSe analysis showed that *Anaerostipes*, *Bifidobacterium*, *Blautia*, *Catenibacterium*, *Collinsella*, *Enterococcus*, *Erysipelatoclostridium*, *Holdemanella*, *Lachnospiraceae UCG 004*, *Megamonas*, *Roseburia*, *Streptococcus*, and *Veillonella* were discriminative genera of fermentation in the presence of Synergy 1 when compared to cellulose (**Figure 4-17**).

4.4.4.3 Effects on short chain fatty acid and branched chain fatty acid production

Fucus vesiculosus whole seaweed (FVWS)

When compared to cellulose, fermentation in the presence of FVWS was associated with significantly higher concentrations of acetic acid after 24 hrs ($p = 0.048$), 36 hrs ($p < 0.0001$), and 48 hrs ($p < 0.0001$) (**Figure 4-18**); of propionic acid after 24 hrs ($p = 0.021$) and 48 hrs ($p = 0.049$) (**Figure 4-19**); of isobutyric acid after 24 hrs ($p = 0.0001805$) (**Figure 4-24**); of isovaleric acid after 24 hrs ($p = 0.002$), 36 hrs ($p = 0.038$), and 48 hrs ($p = 0.02$) (**Figure 4-25**); and of total BCFA after 24 hrs ($p = 0.000175$), 36 hrs ($p = 0.016$), and 48 hrs ($p = 0.002$) (**Figure 4-26**).

Fucus vesiculosus polysaccharide-rich extract (FVE)

When compared to cellulose, fermentation in the presence of FVE resulted in significantly higher concentrations of acetic acid after 24 hrs (p = 0.026), 36 hrs (p = 0.015), and 48 hrs (p < 0.0001) (**Figure 4-18**); and of propionic acid after 24 hrs (p = 0.048) (**Figure 4-19**).

Laminaria digitata whole seaweed (LDWS)

When compared to cellulose, fermentation in the presence of LDWS resulted in significantly higher concentrations of acetic acid after 10 hrs (p = 0.004) (**Figure 4-18**); of butyric acid after 24 hrs (p = 0.002) and 36 hrs (p = 0.024) (**Figure 4-20**); of valeric acid after 24 hrs (p = 0.002) (**Figure 4-21**); of hexanoic acid after 24 hrs (p = 0.037) (**Figure 4-22**); of total SCFA after 24 hrs (p = 0.000427) and 36 hrs (p = 0.011) (**Figure 4-23**); of isobutyric acid after 24 hrs (p < 0.0001), 36 hrs (p = 0.001), and 48 hrs (p = 0.007) (**Figure 4-24**); of isovaleric acid after 24 hrs (p < 0.0001), 36 hrs (p = 0.001), and 48 hrs (p = 0.002) (**Figure 4-25**); and of total BCFA after 24 hrs (p < 0.0001), 36 hrs (p = 0.0001172), and 48 hrs (p = 0.00013788) (**Figure 4-26**).

Laminaria digitata polysaccharide-rich extract (LDE)

The concentration of acetic acid was significantly higher in vessels fermented in the presence of LDE when compared to cellulose after 5 hrs (p = 0.006), however, the acetic acid concentration was significantly greater in vessels fermented in the presence of LDE when compared to cellulose at 0 hrs (p = 0.00015) (**Figure 4-18**). Isobutyric acid was significantly higher in vessels fermented in the presence of LDE after 24 hrs when compared to cellulose (p = 0.032) (**Figure 4-24**).

Synergy 1

There was no significant difference in the concentration of individual SCFA or total SCFA, nor individual BCFA or total BCFA, at any time point between vessels fermented in the presence of Synergy 1 when compared to cellulose.

4.5 Discussion

The use of *in vitro* fermentation experiments as a proxy for colonic fermentation by the gut microbiota is a well-established approach to evaluate prebiotic candidates for their ability to modulate microbial composition and metabolic output (Pham and Mohajeri, 2018). This study aimed to determine the prebiotic potential of the brown seaweeds *Fucus vesiculosus* and *Laminaria digitata*, and polysaccharide-rich extracts of *F. vesiculosus* and *L. digitata*, and to characterise how these substrates modulated the microbial composition of the human faecal microbiota. The data obtained showed that neither *F. vesiculosus*, *L digitata*, or their polysaccharide extracts, impacted the targeted microbial markers of prebiotic activity (qPCR - *Bifidobacterium* spp. and *Lactobacillus* spp.), nor the purported marker of microbial diversity (16S rRNA amplicon sequencing). However, notable changes in short chain fatty acid concentrations occurred when compared to cellulose. This suggests that the brown seaweeds *Fucus vesiculosus* and *Laminaria digitata* contain components that are fermented by human gut microbiota populations to produce health associated SCFA.

There is increasing interest in the application of brown seaweeds as functional food ingredients owing to the purported health benefits of their non-digestible carbohydrate components (Brown et al., 2014; Gueven et al., 2020; Wright et al.,

2019). Such components include alginate (1,4-linked α-L-guluronic acid and β-D-mannuronic acid (Vera et al., 2011), fucoidan (sulphated 1,2- or 1,3- or 1,4- α-L-fucose (Jiao et al., 2011; Vera et al., 2011; Zaporozhets et al., 2014)), and laminarin (1,3- or 1,6- β-D-glucose (Kadam et al., 2015)) polysaccharides. Brown seaweed carbohydrates resist endogenous digestion and are hypothesised to be microbiota-accessible carbohydrates for saccharolytic fermentation by the colonic microbiota (O'Sullivan et al., 2010). For example, *Bacteroides* spp. are reported to expresses the alginate lyase and laminarinase carbohydrate active enzymes (CAZymes), which break down alginate and laminarin polymers, respectively (Li et al., 2016; Mathieu et al., 2018; Salyers et al., 1977).

In the present study, both FVWS and FVE treatment resulted in significantly higher concentrations of acetic and propionic acids when compared to cellulose. While acetic acid, butyric acid, and total SCFA were significantly higher following LDWS treatment when compared to cellulose. These data are in partial agreement with another *in vitro* fermentation of the brown seaweed kelp, *Ecklonia radiata*, where the whole seaweed and a crude fraction significantly increased acetate, propionate, butyrate and total SCFA concentrations after 24 hrs when compared to the cellulose control (Charoensiddhi et al., 2017).

Acetic acid concentrations were significantly higher in LDE treated vessels compared to cellulose after 5 hrs, but not thereafter, which is in contrast to a previous *in vitro* fermentation of another *L. digitata* polysaccharide extract (Strain et al., 2019). This observation may be a false positive because acetic acid concentrations were significantly greater in vessels containing LDE when compared to cellulose at 0 hrs. Given that a previous *in vitro* fermentation study showed that

laminarin content reduced from 100 % to 6.65 % after 24 hrs (Devillé et al., 2007), the apparent low fermentability of LDE in the present study may be owing to low amounts of laminarin within the extract.

The lag effect of SCFA production observed in vessels treated with FVWS, FVE, and LDWS, has been previously described for laminarin and alginate substrates (Kuda et al., 2005), and is in agreement with previous *in vitro* fermentation studies of brown seaweeds and their extracted polysaccharides (Chen et al., 2018; Devillé et al., 2007; Fu et al., 2018; Li et al., 2016; Rodrigues et al., 2016; Seong et al., 2019; Strain et al., 2019). The lag phase of bacterial growth is understood to prioritise carbon source utilization enzyme expression over genes involved in biomass accumulation (Schultz and Kishony, 2013). Thus, the *ex vivo* microbiota in the present study may undergo a metabolic adaptation period upon exposure to a novel carbon source. This may have implications on the optimal dose and duration of dietary intervention studies in human participants (Leeming et al., 2019).

Previous *in vitro* fermentation studies which have explored the prebiotic potential of brown seaweeds and their extracted complex polysaccharides and oligosaccharides have demonstrated the stimulation of bacterial genera such as *Bifidobacterium, Bacteroides*, *Coprococcus*, *Fecalibacterium*, *Lactobacillus, Parabacteroides, Parasutterella, Roseburia* and *Ruminococcus* (Bai et al., 2017; Charoensiddhi et al., 2017, 2016; Devillé et al., 2007; Fu et al., 2018; Li et al., 2016; Seong et al., 2019; Strain et al., 2019). In the present study, FVWS treatment elicited few changes to the bacterial community when compared to cellulose. Although LEfSe analysis indicated that FVWS fermentation was associated with the butyrate-producing genera *Butyricimonas* after 24 hrs (Sakamoto et al., 2014),

there were also associations with opportunistic pathogens such as *Citrobacter,* *Enterobacter,* and *Klebsiella*. Thus, it is necessary to investigate the impact of dietary consumption of *Fucus vesiculosus* seaweed *in vivo*, to understand whether this seaweed stimulates the growth of potential pathogens, or whether the observation in the current study is associated with the use of an *in vitro* fermentation model. Further, there is also a knowledge gap regarding the catabolism of complex carbohydrates by pathobionts, which may have wider implications concerning the use of microbiota accessible carbohydrates as supplements.

Fucus vesiculosus polysaccharide-rich extract (FVE) treatment was associated with the stimulation of several genera which catabolise dietary complex carbohydrates, including those within the *Lachnospiraceae*, *Prevotellaceae*, and *Ruminococcaceae* families (Biddle et al., 2013; Kovatcheva-Datchary et al., 2015). Previous *in vitro* fermentation and *in vivo* animal studies have also reported increases in these bacterial families (An et al., 2013; Strain et al., 2019; Umu et al., 2015). The families *Ruminococcaceae* and *Lachnospiraceae* contain common gut commensals in individuals with fibre-rich and plant-based diets (Lagier et al., 2012; Schnorr et al., 2014), owing to multiple CAZymes which harvest energy from complex polysaccharides to promote their survival (Biddle et al., 2013; Esquivel-Elizondo et al., 2017). Further, many of the major butyrate producers in the human gut are members of the *Ruminococcaceae* and *Lachnospiraceae* families (Vital et al., 2017). Thus, more in-depth microbial characterisation at the functional and strain level may be useful to understand how brown seaweed glycans are utilised by *Lachnospiraceae* and *Ruminococcaceae.*

FVE also stimulated the genera *Phascolarctobacterium*, *Oscillospira*, and *Faecalibacterium*, which corroborates previous findings following the *in vitro* fermentation of an *Ascophyllum nodosum* sulphated polysaccharide extract (Chen et al., 2018). Considering that *Phascolarctobacterium* produce acetate and propionate (Wu et al., 2017), and that *Faecalibacterium prausnitzii* is a candidate next generation probiotic and a major constituent of the adult human gut microbiota population (Martín et al., 2018), further experiments to characterise the effect of the sulphated polysaccharide, fucoidan, obtained from *Fucus* spp. may be warranted.

Other SCFA-producing microbial genera associated with FVE fermentation were *Anaerostipes* (propionic acid) (Engels et al., 2016), *Eubacterium hallii* (propionic acid and butyric acid) (Flint et al., 2012), *Anaerotruncus* (butyric acid) (Le Chatelier et al., 2013) and *Pseudobutyrivibrio* (butyric acid) (Bailey et al., 2011). *Pseudobutyrivibrio* are suggested to reduce gut inflammation in mice (Bailey et al., 2011; Neto and O'Toole, 2016), while a greater abundance of *Anaerotruncus* and has been reported in lean individuals when compared to obese individuals (Hou et al., 2017; Mancabelli et al., 2017).

Similar to FVE, *Laminaria digitata* whole seaweed (LDWS) treatment was associated with genera from the families *Ruminococcaceae* and *Lachnospiraceae*, which corroborates previous evidence from *in vitro* fermentation and *in vivo* rodent studies (Choa An et al., 2013; Shang et al., 2016; Strain et al., 2019). LDWS also stimulated the genus *Bacteroides*, which utilises a range of dietary glycans and can adapt to the nutritional availability in the colonic environment owing to the

vast number of polysaccharide utilization loci present in their genomes (Benítez-Páez et al., 2017; Patnode et al., 2019; Rios-Covian et al., 2017).

The LDWS-associated changes in SCFA concentration may be attributed to some microbial genera that proliferated throughout the fermentation, including *Veillonella* (propionic acid) (Scheiman et al., 2019), *Lachnospiraceae* (butyric acid) (Tidjani Alou et al., 2016), *Butyricimonas* (butyric acid) (Sakamoto et al., 2014), and *Coprococcus* (butyric acid) (Valles-Colomer et al., 2019).

The stimulation of the genera *Coprococcus* and *Parasutterella* by LDWS was also observed during an *in vitro* fermentation of a *Sargassum thunbergii* polysaccharide extract (Fu et al., 2018). *Parasutterella* are assaccharolytic bacteria and were recently suggested to be involved in bile acid and cholesterol metabolism (Ju et al., 2019), but further understanding of this genera is important to understand any potential benefit of stimulating their growth and metabolism by seaweed glycans.

LDE treatment was associated with the genera *Butyricimonas* and Coprococcus, which were also stimulated by LDWS. Similarly, the stimulation of *Phascolarctobacterium* and *Pseudobutyrivibrio* by LDE was also observed in FVE-treated vessels. This may suggest the presence of common carbohydrate constituents in these three fermentation substrates. The genus *Roseburia* was also associated with LDE fermentation, which corroborates previous reports of increased *Roseburia* spp. populations following the *in vitro* fermentation of a *Sargassum thunbergii* polysaccharide extract (Fu et al., 2018), and in pigs fed alginate (5.14 % (w/w) for 84 days) (Umu et al., 2015). *Roseburia* are a polysaccharide utilising genus of the *Lachnospiraceae* family that produce butyrate and have an anti-inflammatory role to maintain gut homeostasis (Duncan et al.,

2006; La Rosa et al., 2019). *Roseburia inulinivorans* is reported to contain fucosidase enzymes for fucose utilisation (Scott et al., 2006), while *R. intestinalis* is predicted to specialize in the degradation of plant cell wall matrix polysaccharides (Sheridan et al., 2016).

The average molecular weight of FVE and LDE in the currentstudy was similar (6.2 kDa and 6.1 kDa, respectively), but the total sulphate content, as equivalents of purified fucoidan, varied (FVE = 21.41 ± 0.11 % and LDE = 9.06 ± 0.99 %). This would suggest differences in the amount of fucoidan in each extract. Although it is not possible to ascribe differences in molecular weight and sulphate content to the effects of the FVE and LDE substrates on SCFA and microbial composition in the present study, previous *in vitro* fermentation studies and *in vivo* rodent studies with alginate, fucoidan, and laminarin seaweed polysaccharides indicate that structural heterogeneity and variation of molecular weight impact fermentability (An et al., 2013; Kong et al., 2016; Ramnani et al., 2012; Shang et al., 2016; Terada et al., 1995; Vera et al., 2011; Wang et al., 2006)

Serratia and *Yersinia* (*Yersiniaceae* family) were present in vessels treated with LDE and are genera which contain opportunistic pathogens. Although *Yersinia* are found in low abundance in the human ileum (Le Baut et al., 2018), *Serratia* and *Yersinia* are common food contaminants which can propagate during refrigeration and are reported as residential bacteria of food industry surfaces, including the dairy industry (Barton, 2016; Møretrø and Langsrud, 2017).

The observed significant increase in BCFA concentrations in vessels fermented in the presence of FVWS, LDWS, and LDE suggests that proteolytic fermentation occurred. There is limited knowledge about the effects of BCFA on host health

(Oliphant and Allen-Vercoe, 2019), however, some protein fermentation metabolites such as hydrogen sulphide, ammonia, and *p*-cresol potentially genotoxic (Diether and Willing, 2019). Therefore, further investigation regarding the impact of dietary seaweed and seaweed-derived amino acids, on protein fermentation, is needed.

The data obtained in the present study suggest that FVE and LDE had a lesser impact on SCFA production when compared to other *in vitro* fermentation studies. An obvious cause of variation between such studies is that the faecal microbiota originated from different donors. Another cause of this disparity could be a variation in polysaccharide composition between extracts, thus the heterogenous nature of polysaccharide extracts makes direct comparison difficult. Looking ahead, alternative extraction methodology, such as supercritical CO_2 extraction, may provide a useful tool for homogenous extract production (Chen et al., 2014; Zou et al., 2018).

The synergy of components found in the whole seaweed matrix (e.g. complex polysaccharides, polyphenols, polyunsaturated fatty acids, and carotenoids) may also provide a greater opportunity for bacterial metabolism and energy harvest when compared to extracted components alone. A similar observation was reported for oat bran when compared to beta glucan and polyphenol extracts (Kristek et al., 2019). This highlights the necessity to explore the structure-function relationship of well characterised and purified carbohydrates on bacterial composition and metabolic output during *in vitro* fermentation experiments. Further, a whole genome shogun sequencing approach could provide strain-level

characterisation and information on the functional capacity of the human gut microbiota when exposed to seaweeds and their unique polysaccharides.

4.6 Conclusion

Brown seaweed-derived complex polysaccharides are resistant to human digestive enzymes, which provides scope to explore whether these glycans are accessible substrates for saccharolytic fermentation by the human gut microbiota, conducive to a prebiotic. The data obtained showed that neither *F. vesiculosus*, *L digitata*, or their polysaccharide extracts, impacted the targeted microbial markers of prebiotic activity (qPCR - *Bifidobacterium* spp. and *Lactobacillus* spp.), nor the purported marker of microbial diversity (16S rRNA amplicon sequencing). However, notable changes in short chain fatty acid concentrations occurred when compared to cellulose. This suggests that the brown seaweeds *Fucus vesiculosus* and *Laminaria digitata* contain components that are fermented by human gut microbiota populations to produce health associated SCFA. Based on this study, the brown seaweeds *Laminaria digitata* and *Fucus vesiculosus* show promise about containing putative prebiotic components, however, further investigation is needed to understand how the human gut microbiota degrades and utilises brown seaweeds and their unique polysaccharides.

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4.8 Figures

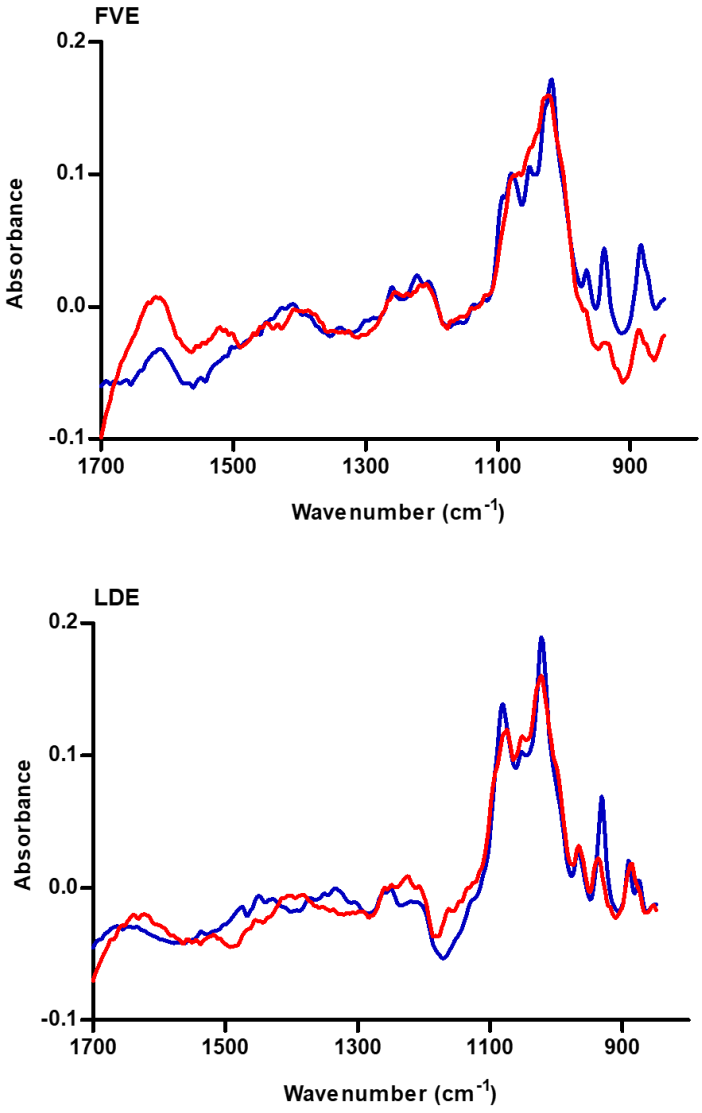

Figure 4-1. Fourier transform infrared spectroscopy (FTIR) spectra was used to provide structural information of the *F. vesiculosus* (FVE) and *L. digitata* (LDE) crude polysaccharide extracts. Blue denotes the extract before *in vitro* digestion and red denotes the extract after *in vitro* digestion.

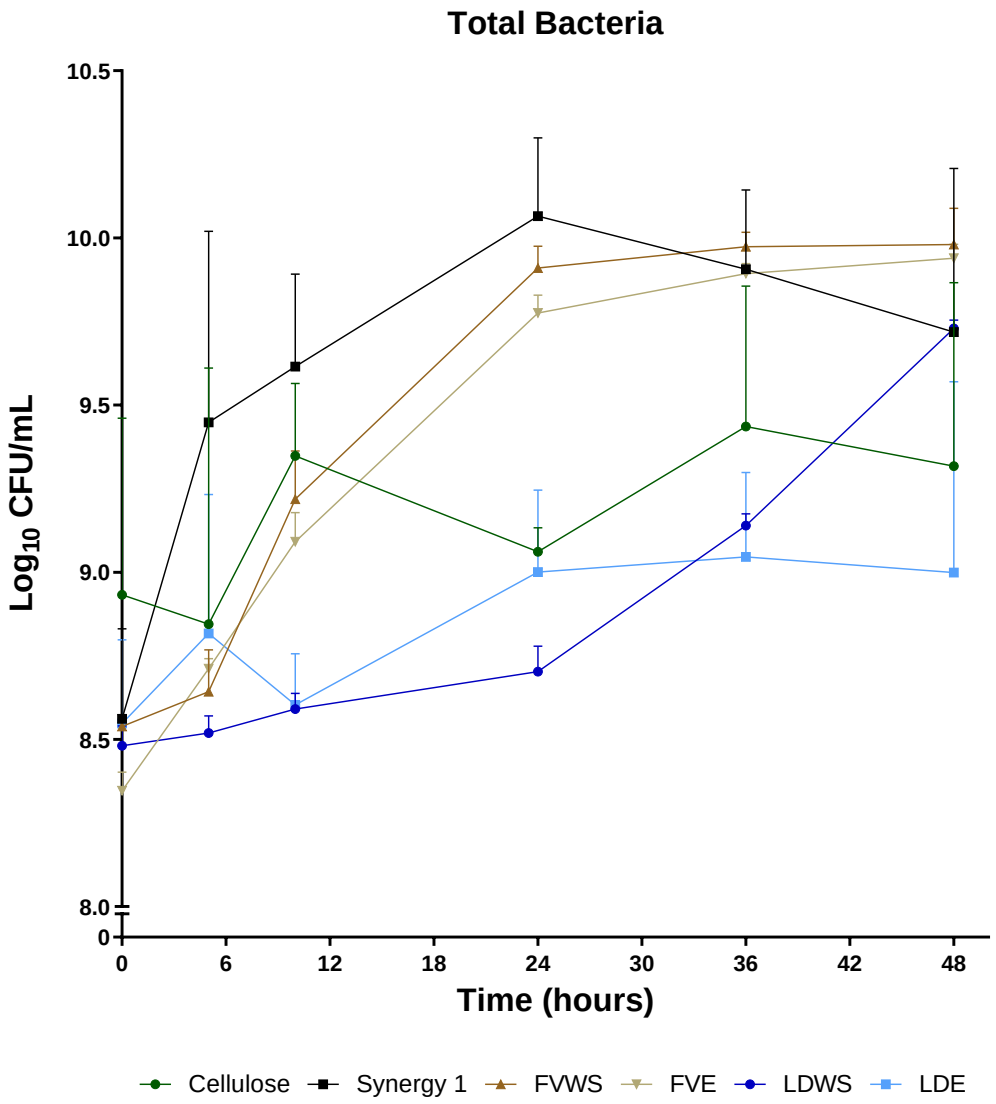

Figure 4-2. qPCR was used to quantify total bacteria from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard deviation of the mean (n = 3). Statistical significance when compared to cellulose was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

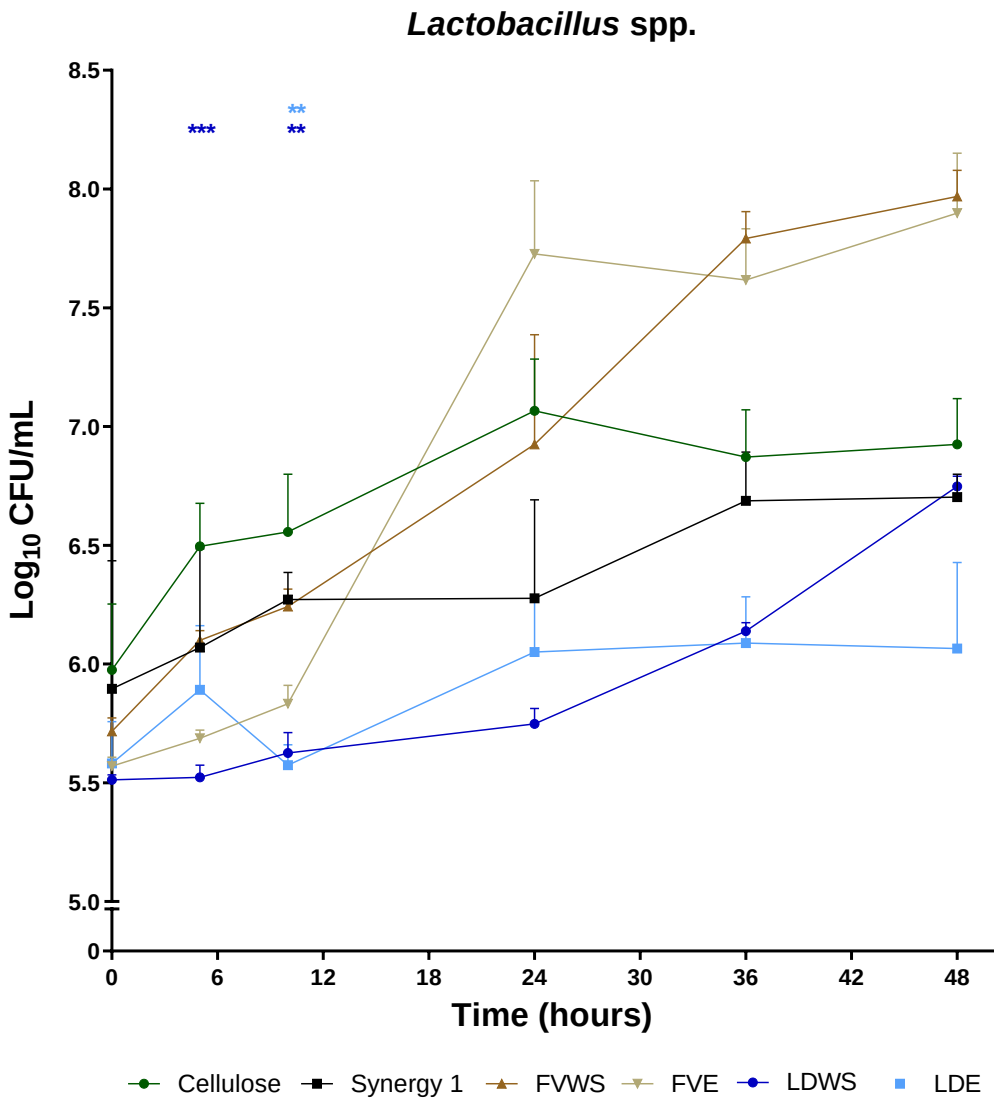

Figure 4-3. qPCR was used to quantify *Lactobacillus* spp., from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard deviation of the mean (n = 3). Statistical significance when compared to cellulose was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

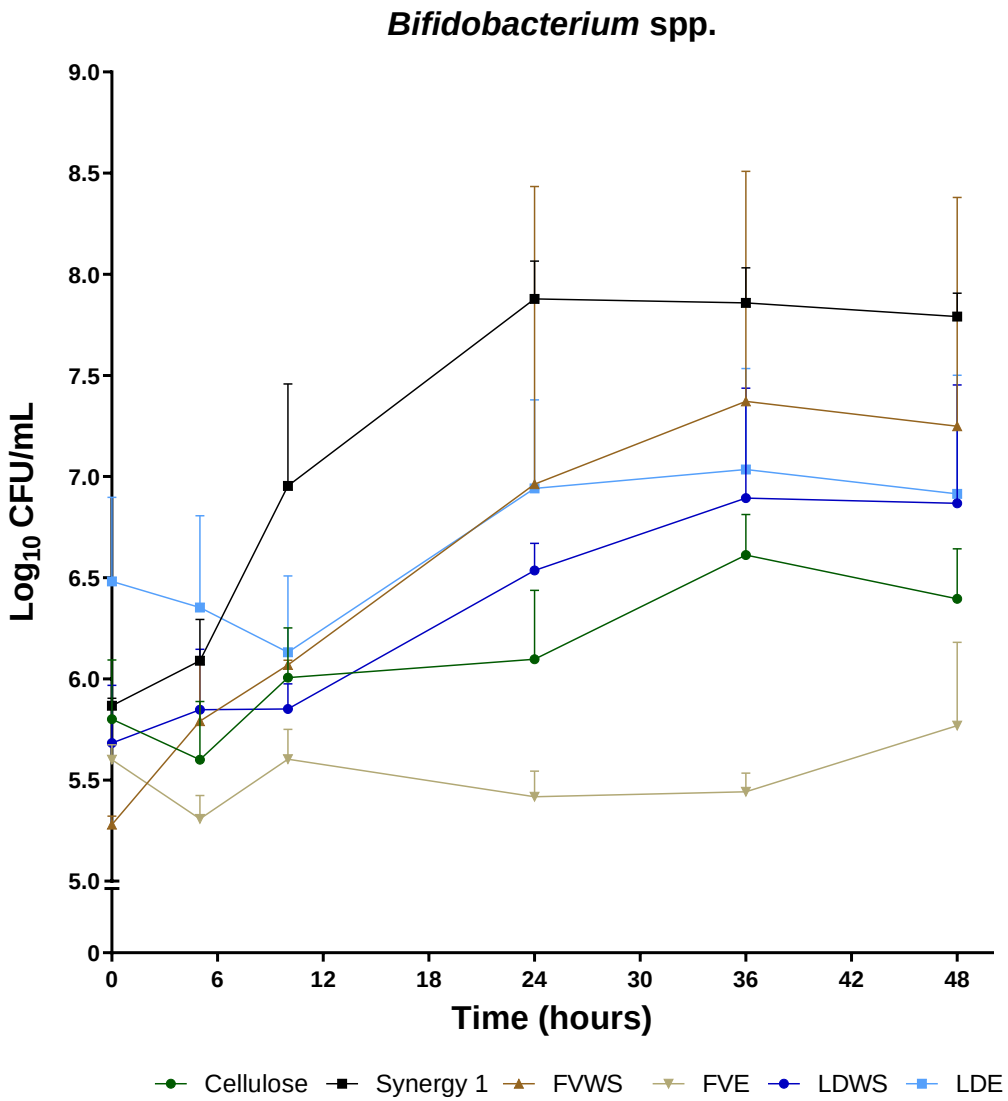

Figure 4-4. qPCR was used to quantify *Bifidobacterium* spp. from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard deviation of the mean (n = 3). Statistical significance when compared to cellulose was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

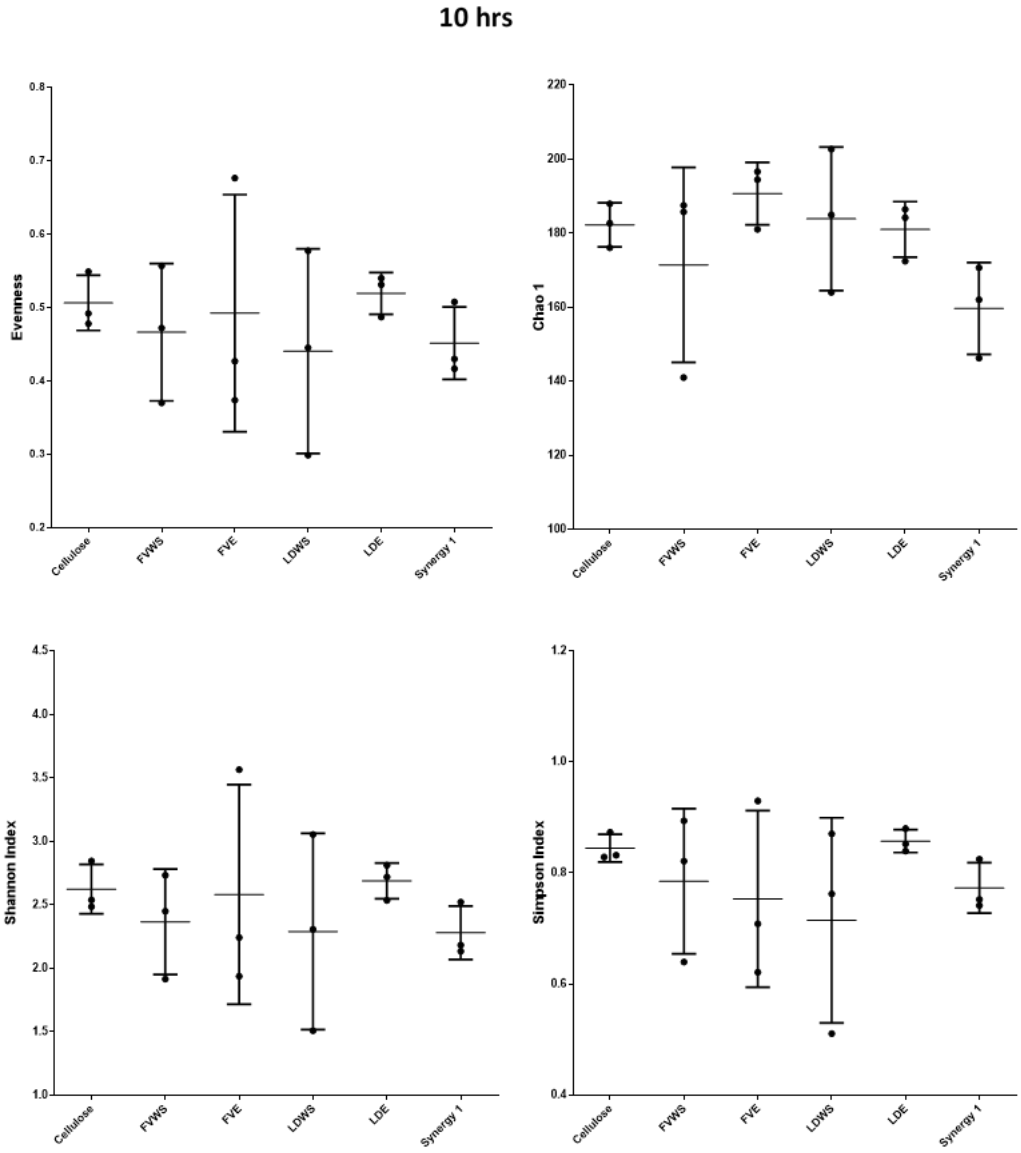

Figure 4-5. Mean alpha diversity indices of Evenness, Chao1, Shannon Index and Simpson Index of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 after 10 hrs. Plots represent individual values and the mean ± one standard deviation of the mean (n = 3). Statistical significance was accepted as $p \leq 0.05$ following a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons.

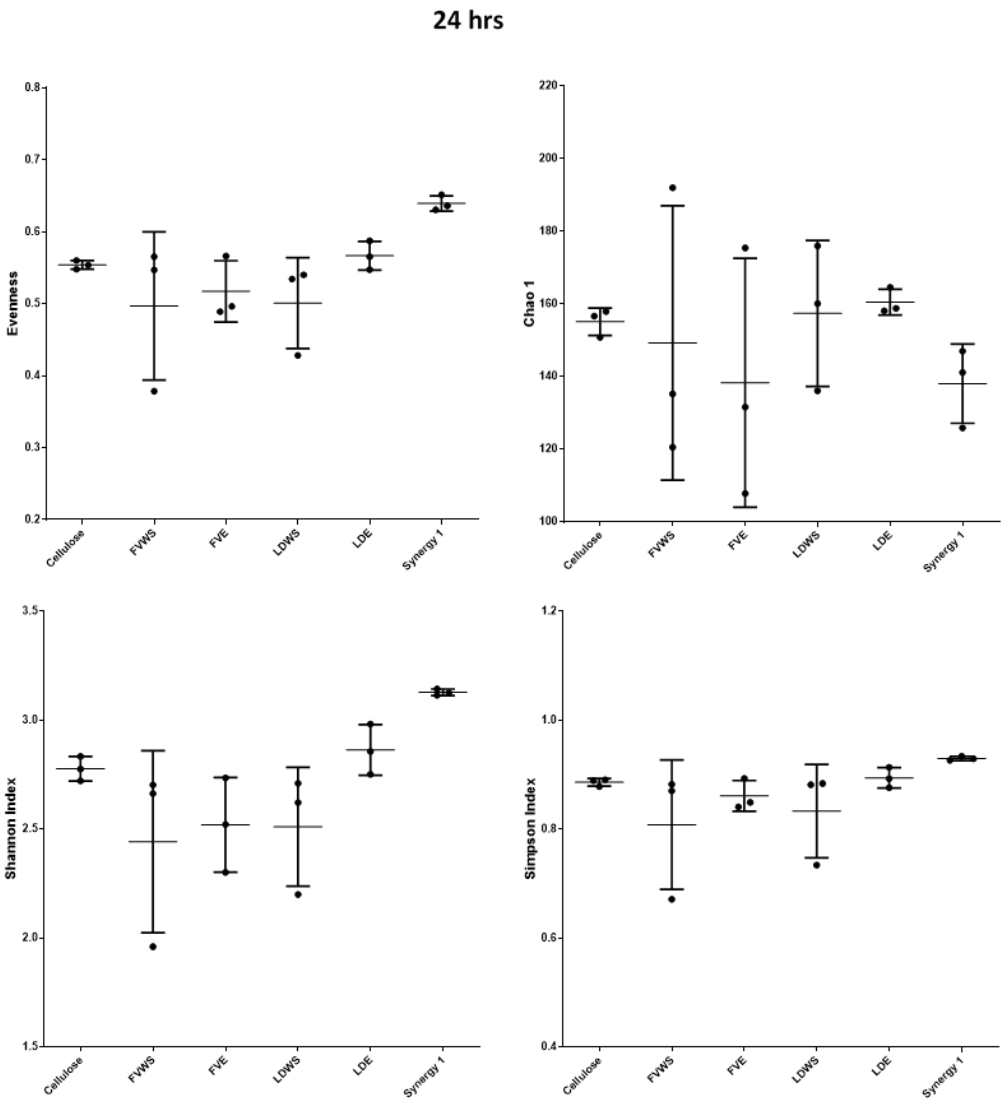

Figure 4-6. Mean alpha diversity indices of Evenness, Chao1, Shannon Index and Simpson Index of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 after 24 hrs. Plots represent individual values and the mean ± one standard deviation of the mean (n = 3). Statistical significance was accepted as $p \leq 0.05$ following a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons.

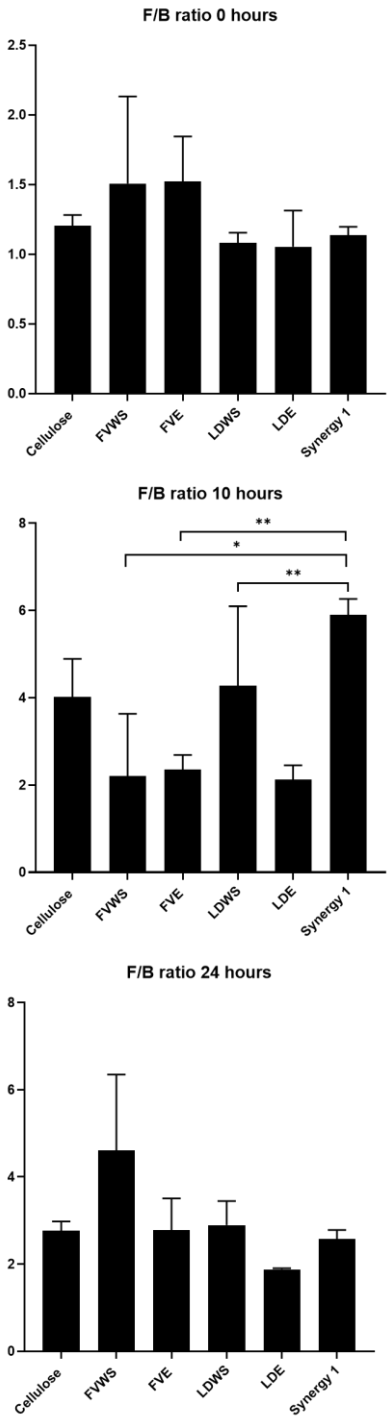

Figure 4-7. Firmicutes to Bacteroidetes (F/B) ratio in fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 after 24 hrs. Plots represent individual values and the mean ± one standard deviation of the mean (n = 3). Statistical significance was accepted as $p \leq 0.05$ following a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$.

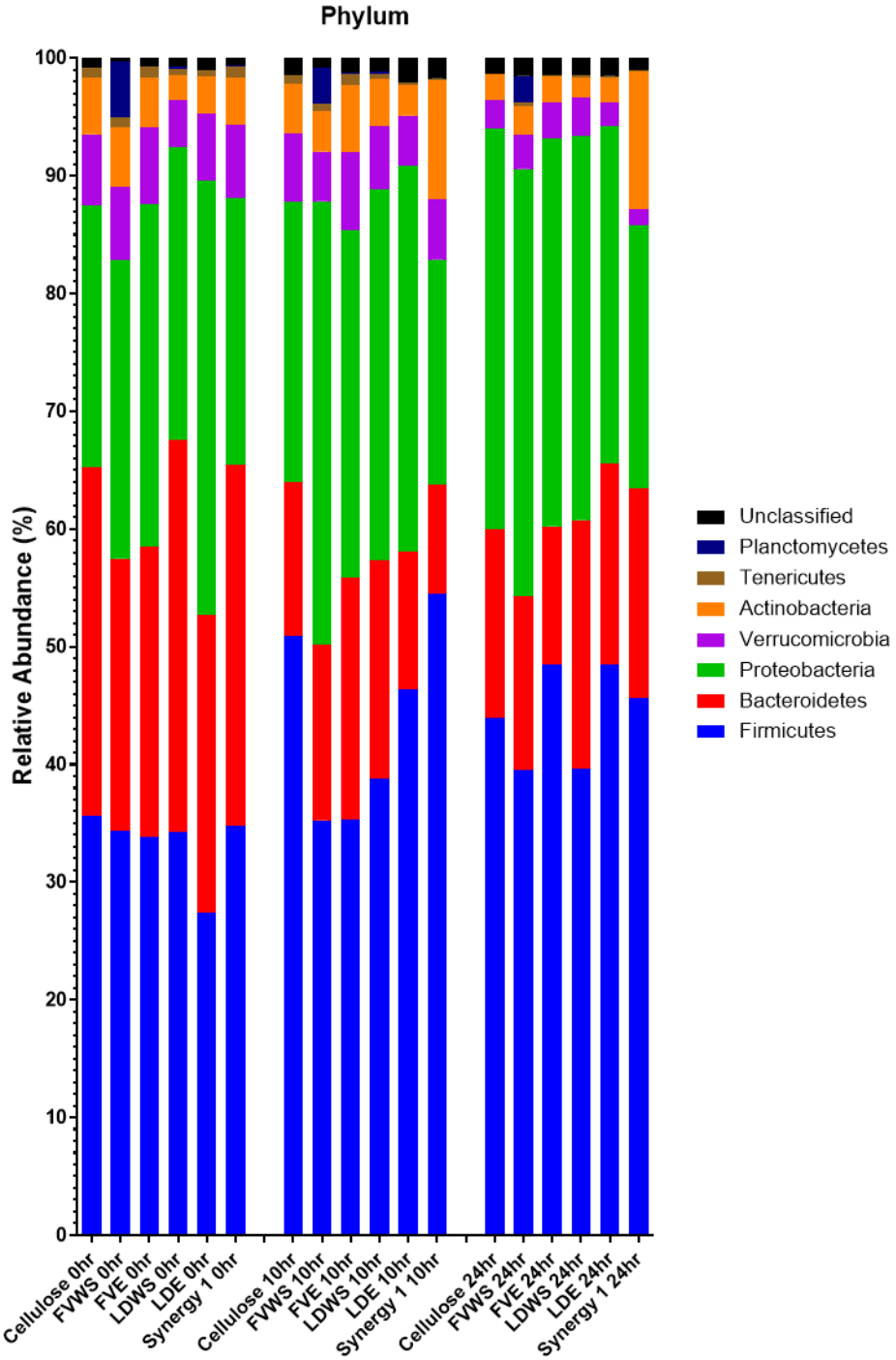

Figure 4-8. Mean relative abundances of bacterial phyla in fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 after 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

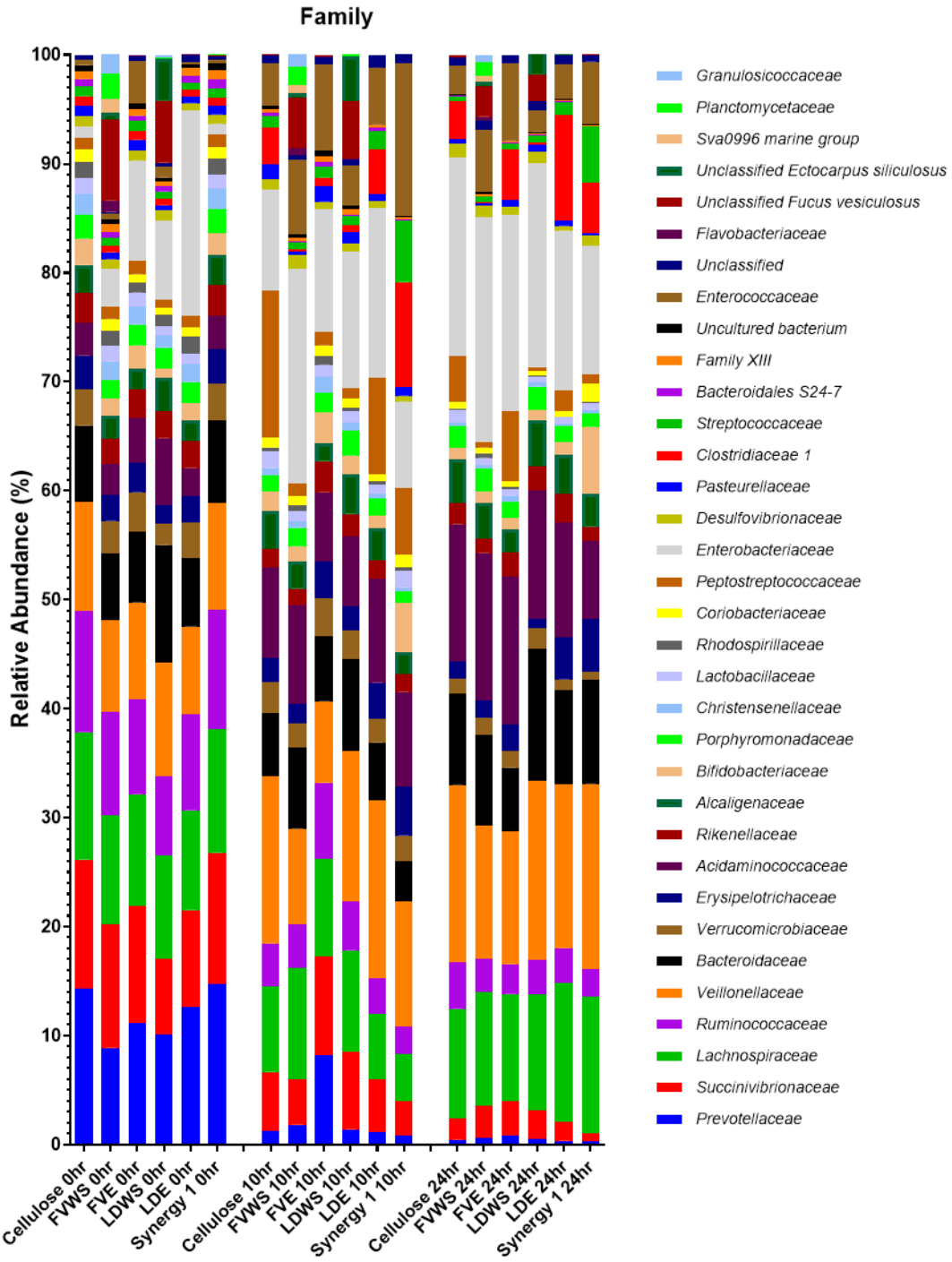

Figure 4-9. Mean relative abundances of bacterial families in fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 after 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

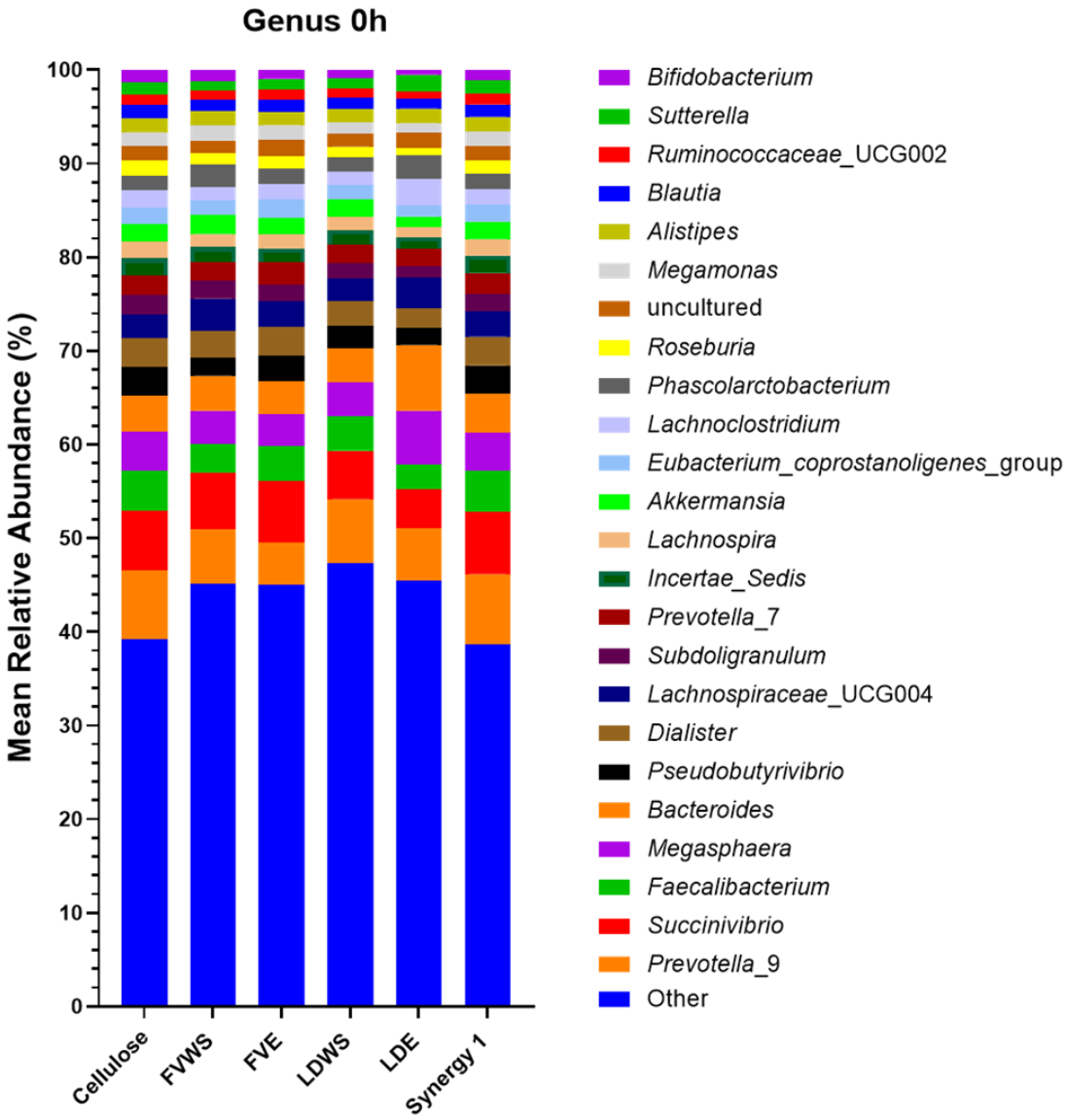

Figure 4-10. Mean relative abundances of bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 at 0 hrs fermentation (n = 3). Individual stacks represent taxa with a mean relative abundance > 1% across all substrates. All remaining genera were assigned a single stack labelled "other". Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

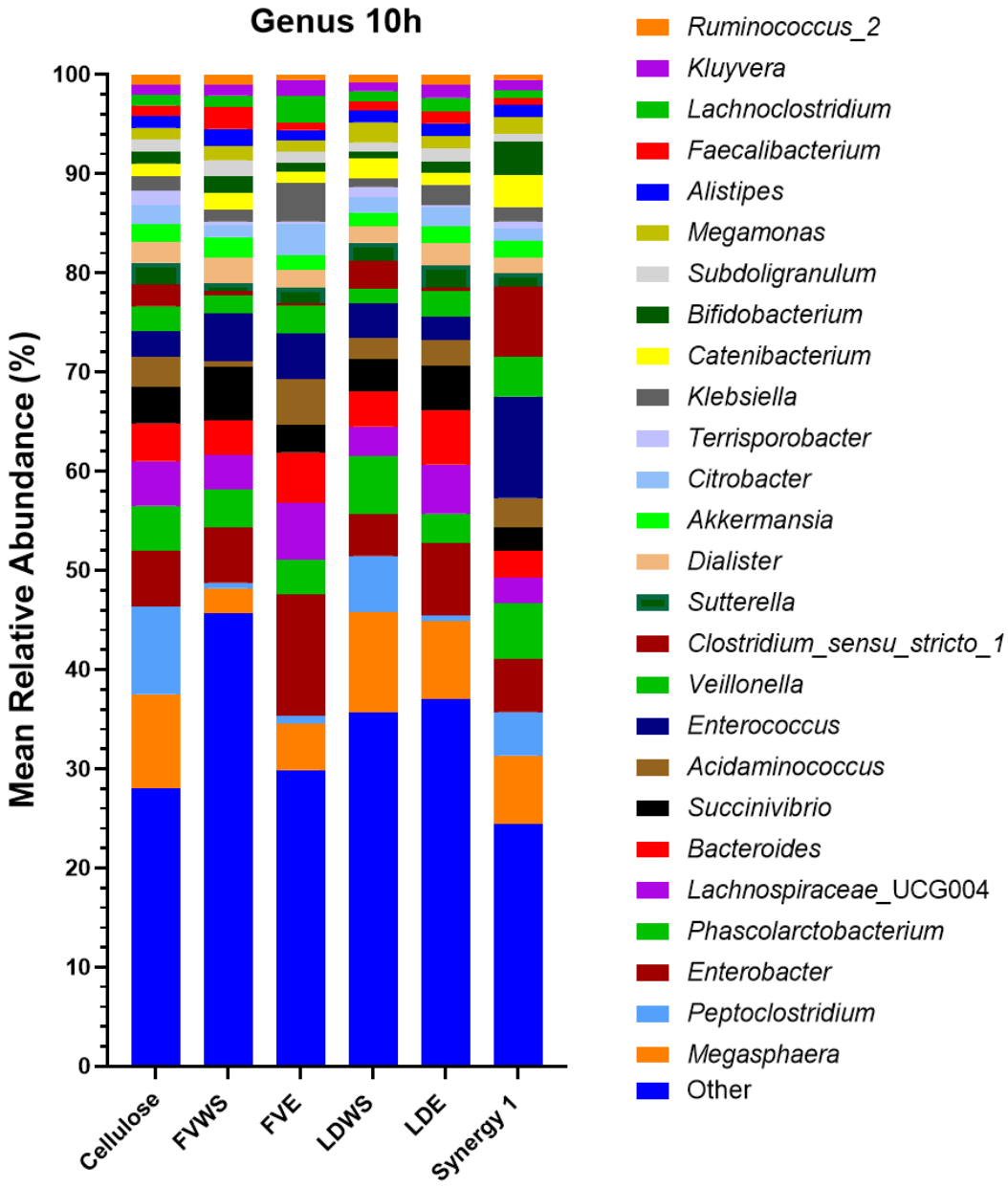

Figure 4-11. Mean relative abundances of bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 at 10 hrs fermentation (n = 3). Individual stacks represent taxa with a mean relative abundance > 1% in the cellulose control. All remaining genera were assigned a single stack labelled "other". Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

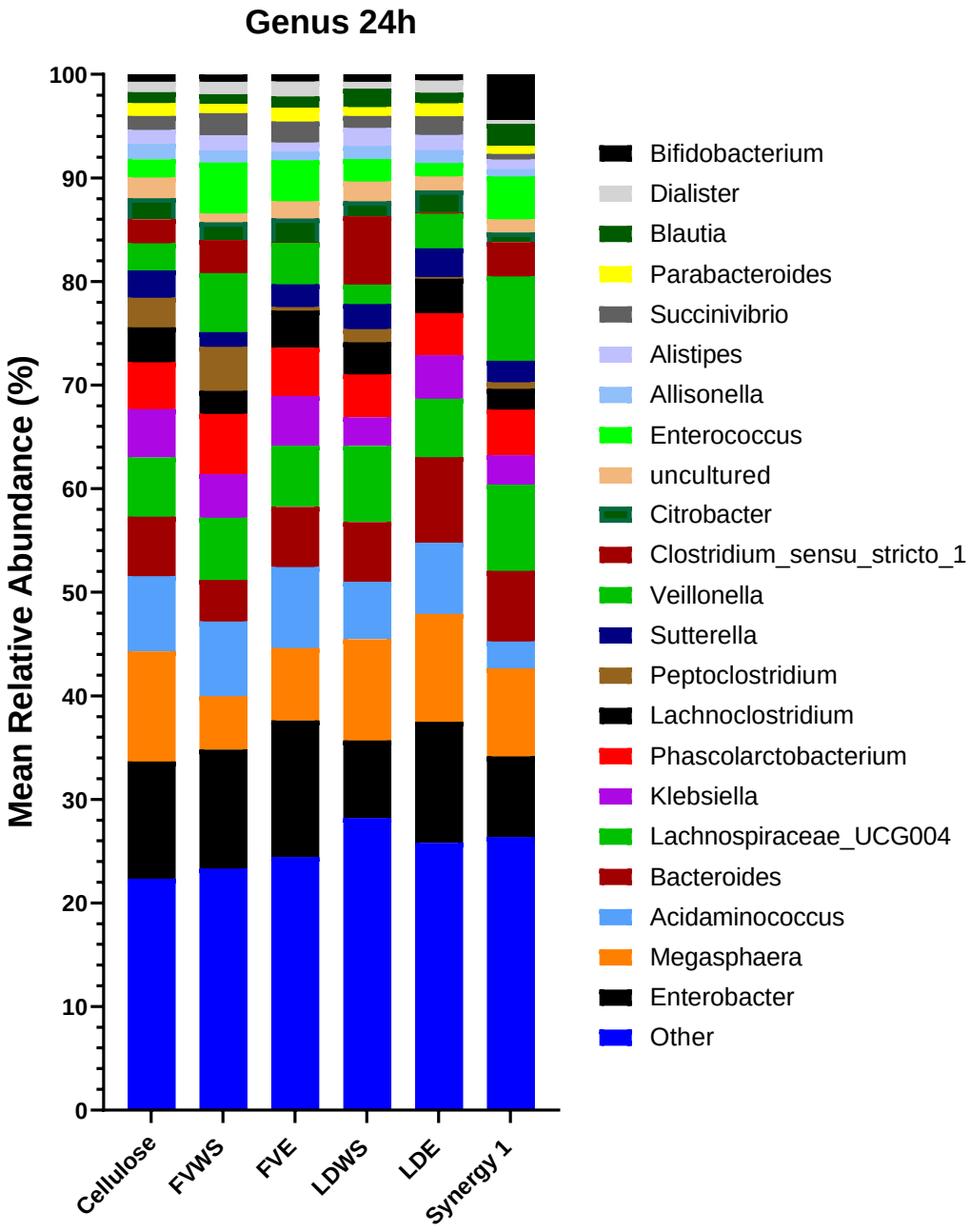

Figure 4-12. Mean relative abundances of bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 at 24 hrs fermentation (n = 3). Individual stacks represent taxa with a mean relative abundance > 1% in the cellulose control. All remaining genera were assigned a single stack labelled "other". Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

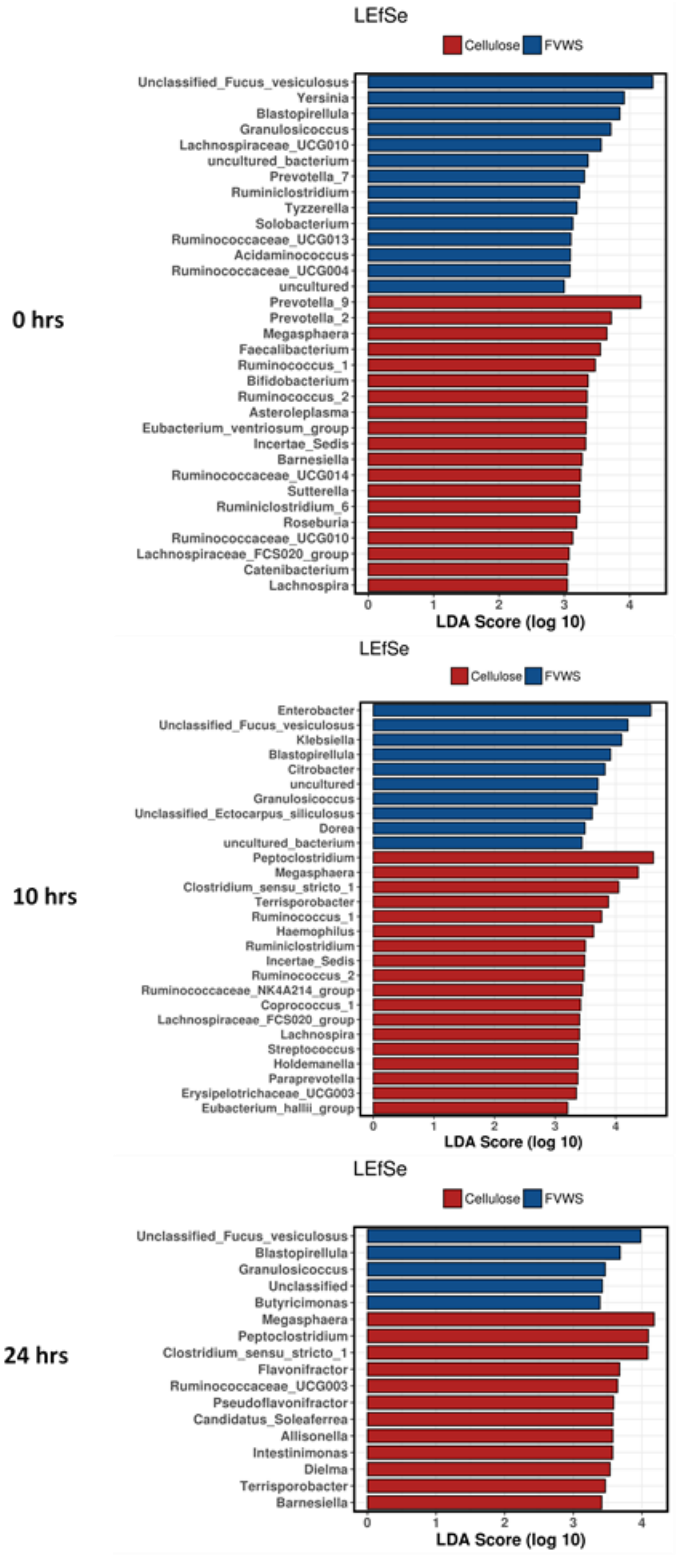

Figure 4-13. LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) *F. vesiculosus* whole seaweed (FVWS) when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

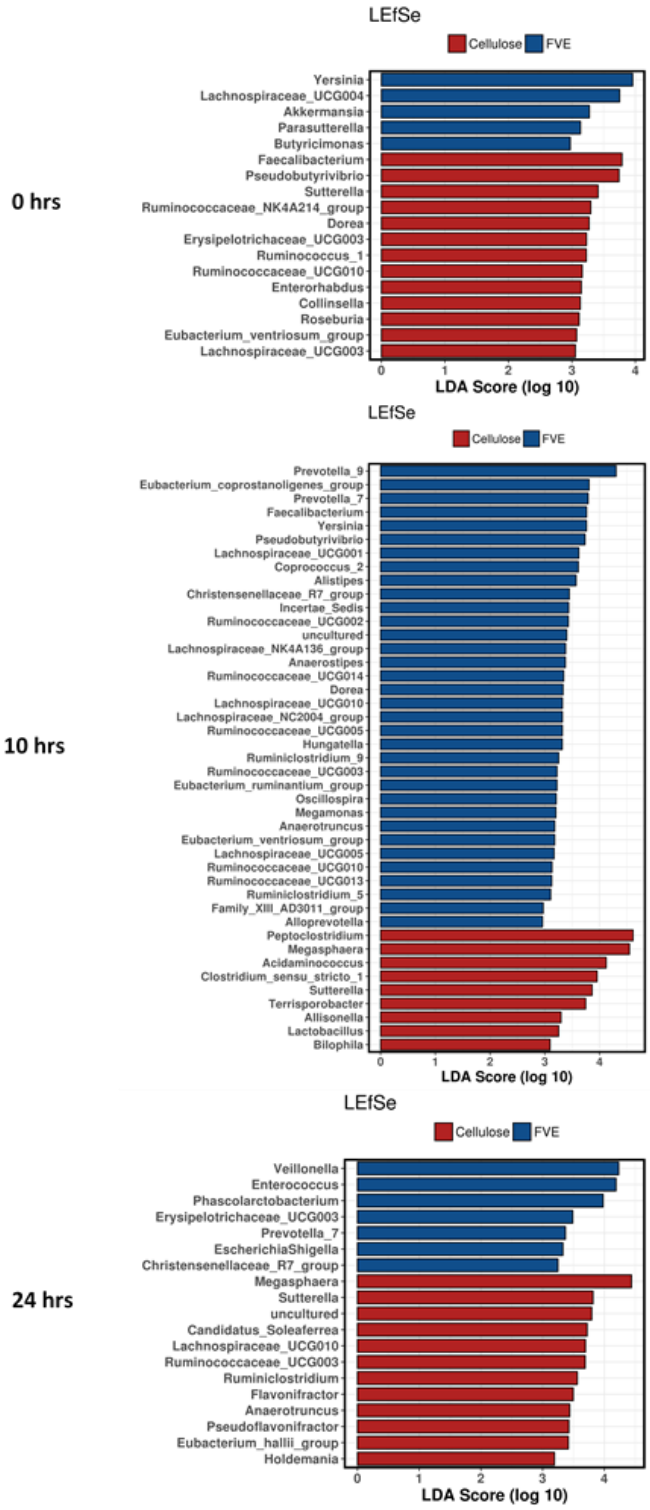

Figure 4-14. LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) *F. vesiculosus* crude polysaccharide extract (FVE) when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

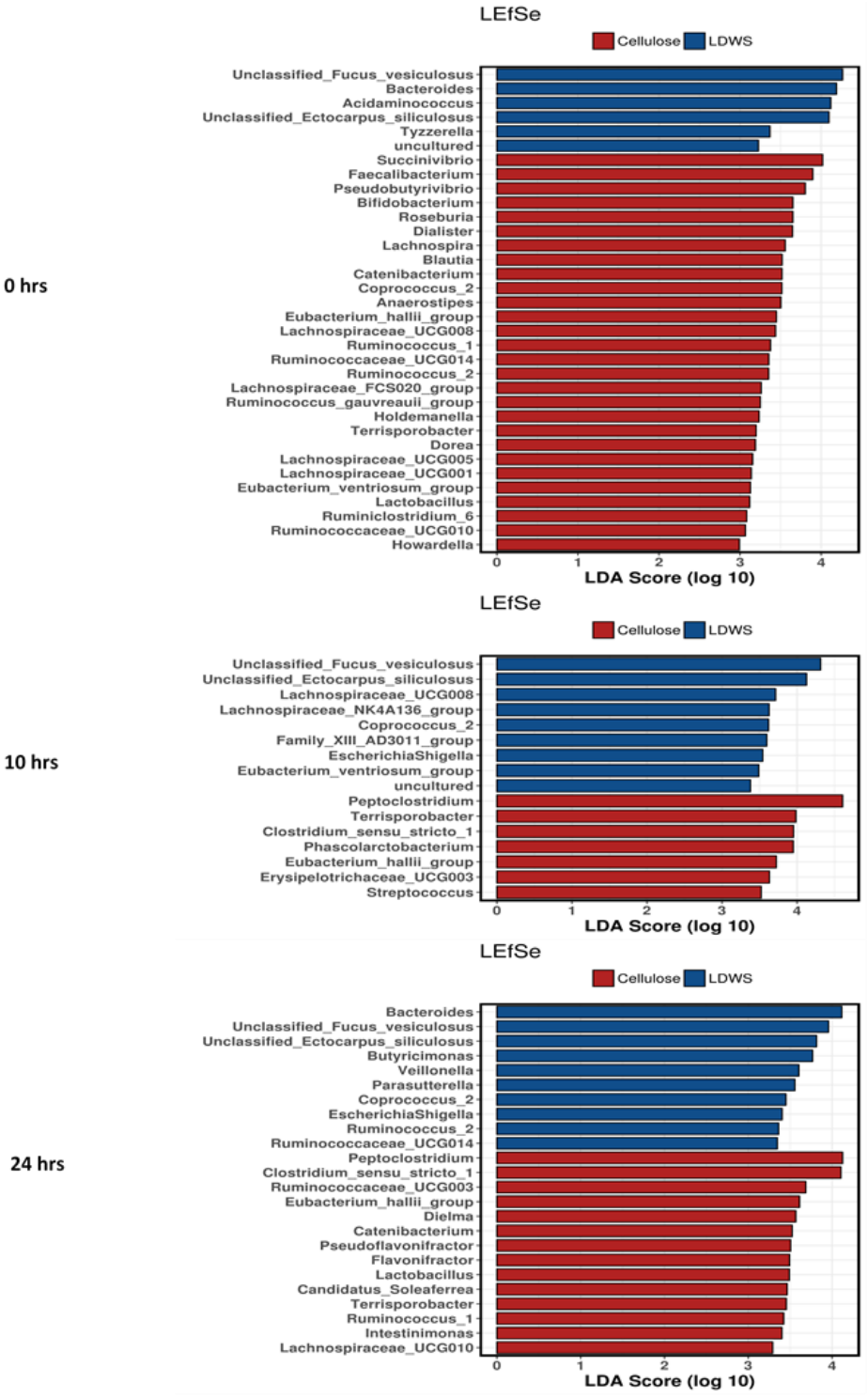

Figure 4-15. LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) *L. digitata* whole seaweed (LDWS) when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

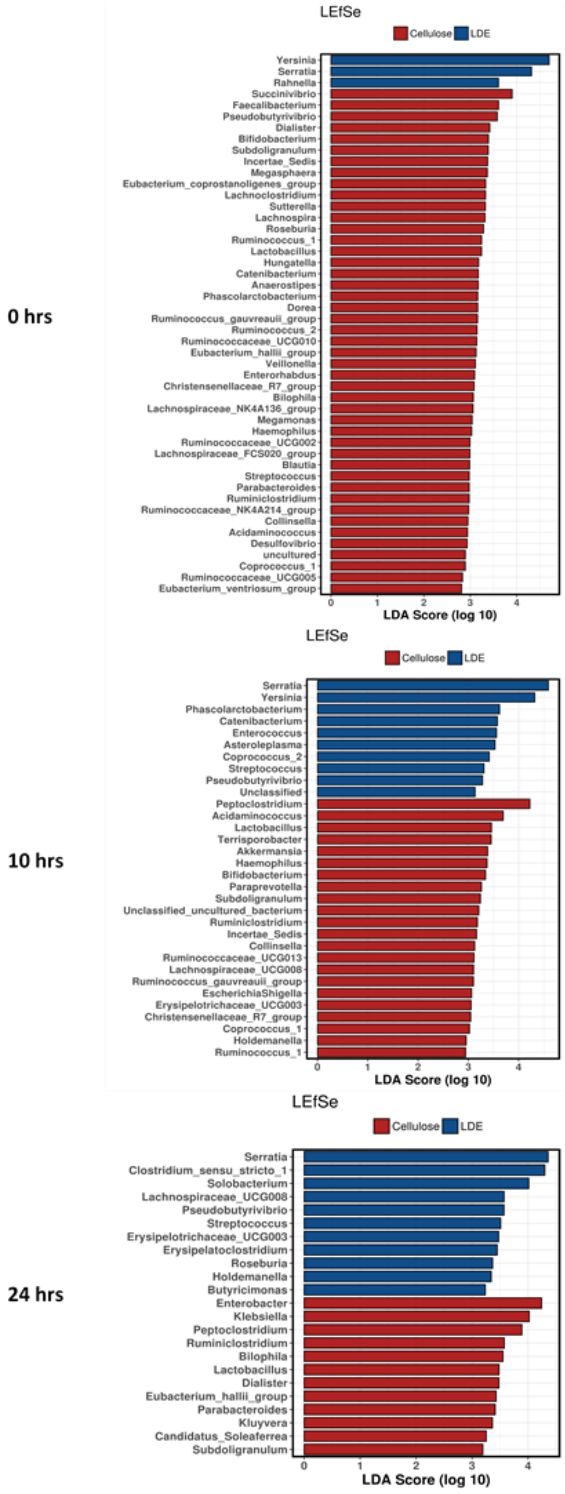

Figure 4-16. LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) *L. digitata* crude polysaccharide extract (LDE) when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

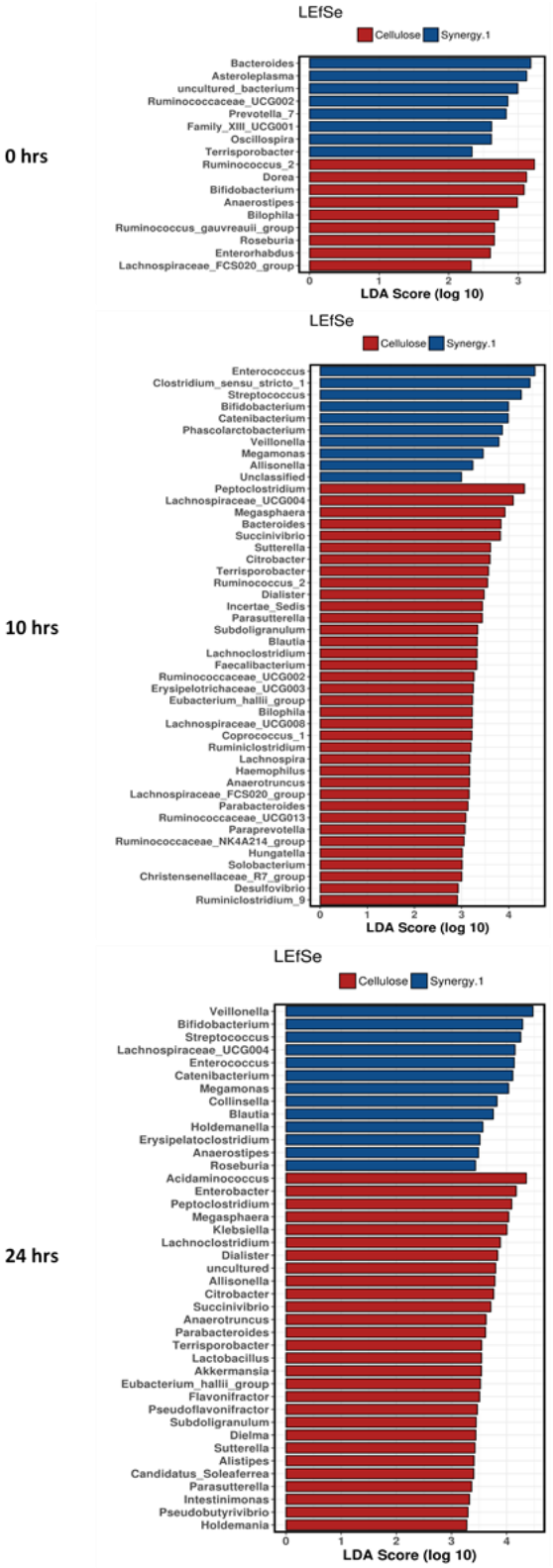

Figure 4-17. LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Synergy 1 when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

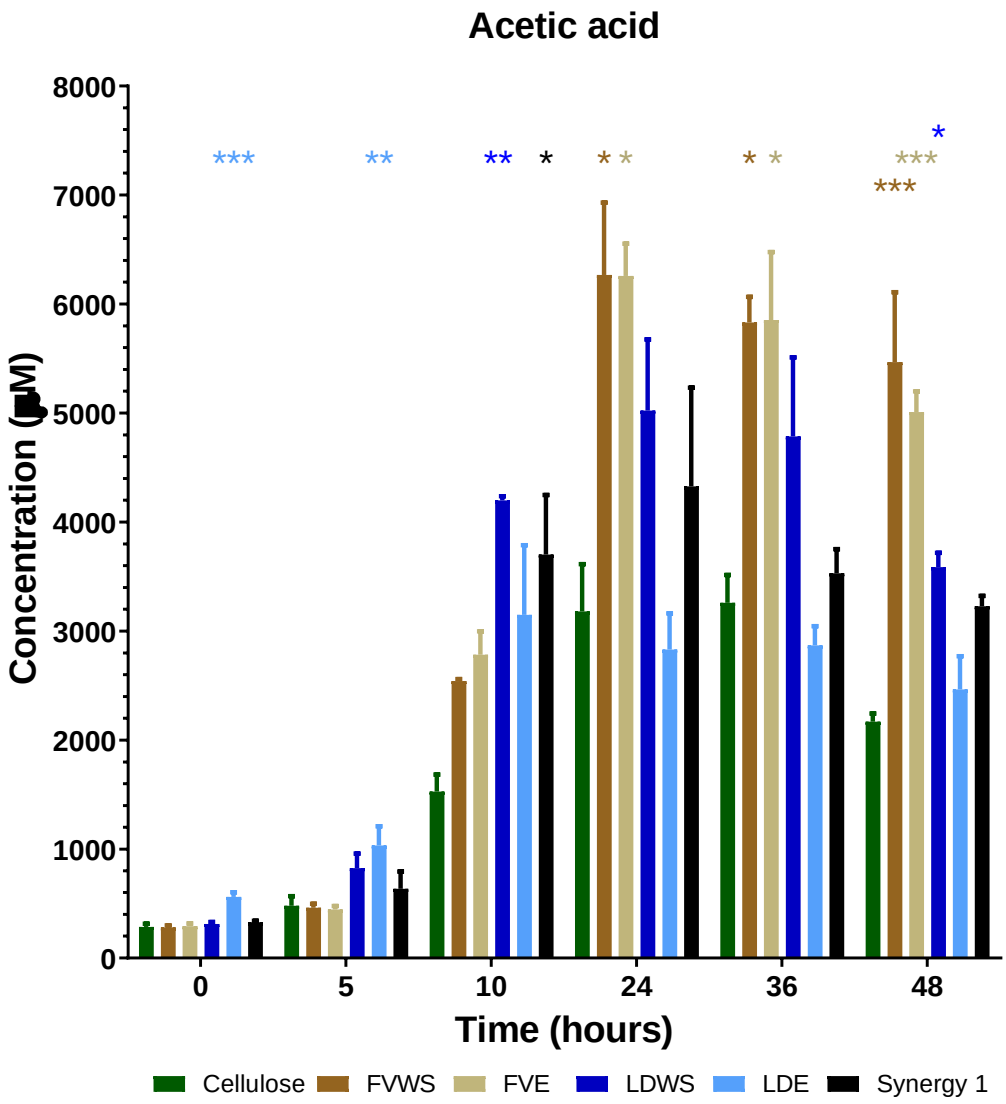

Figure 4-18. Acetic acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

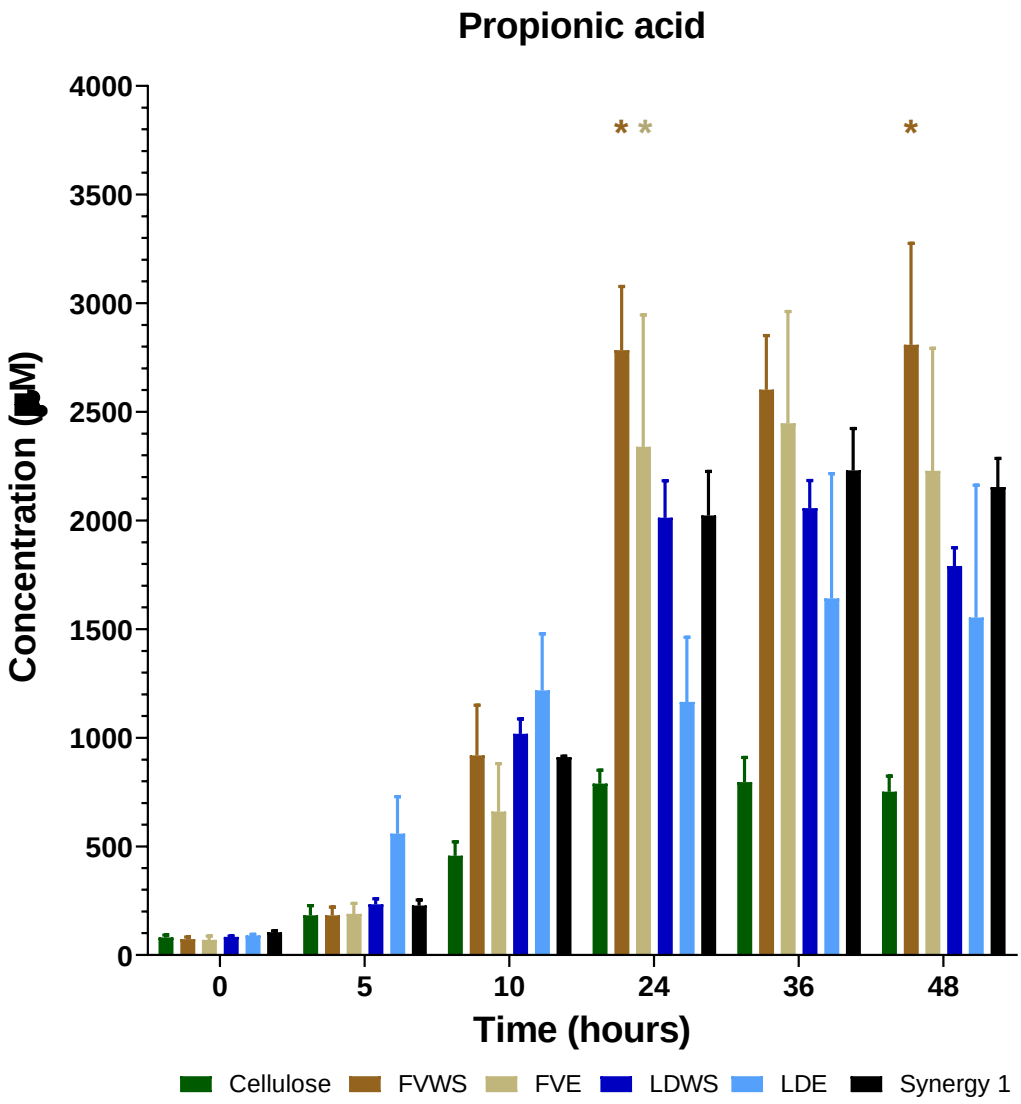

Figure 4-19. Propionic acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

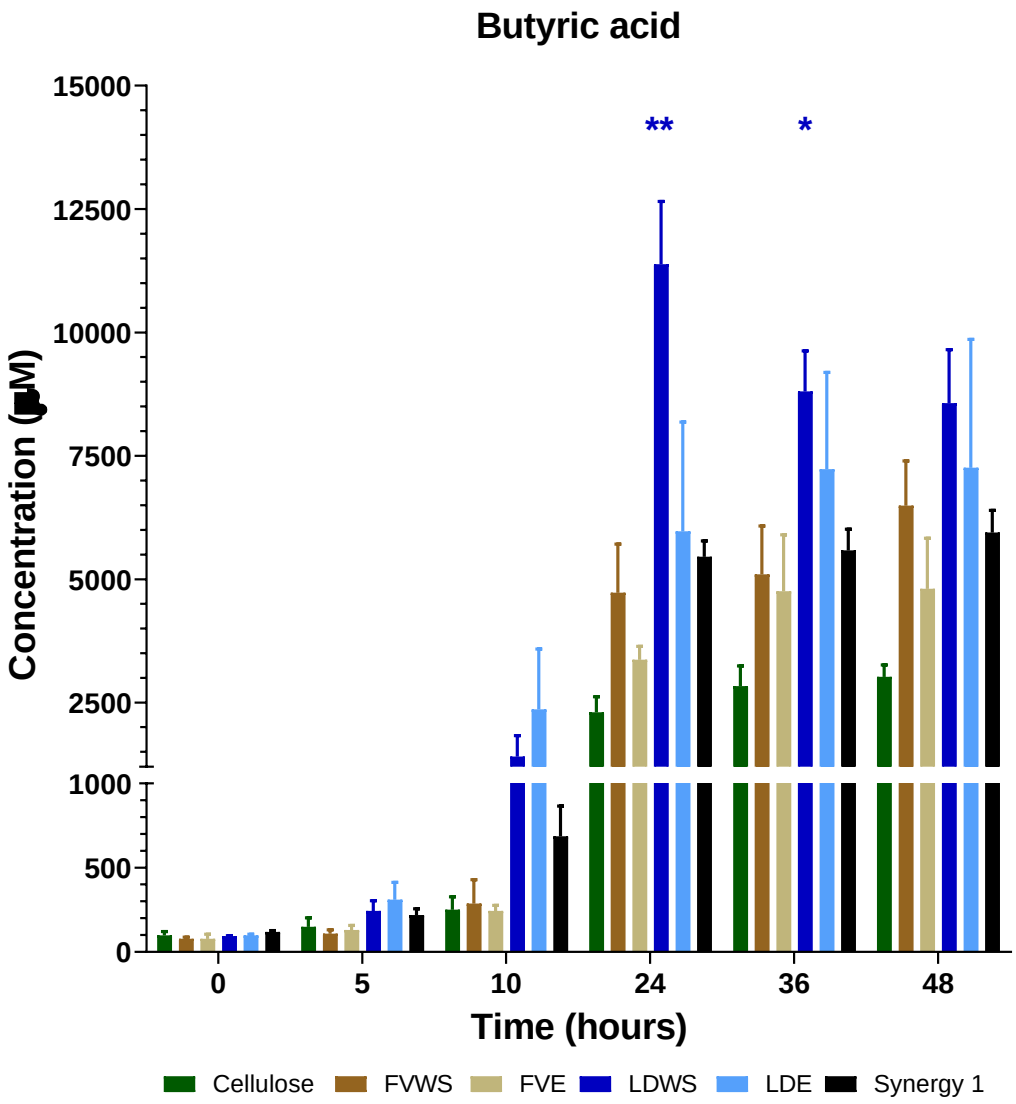

Figure 4-20. Butyric acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

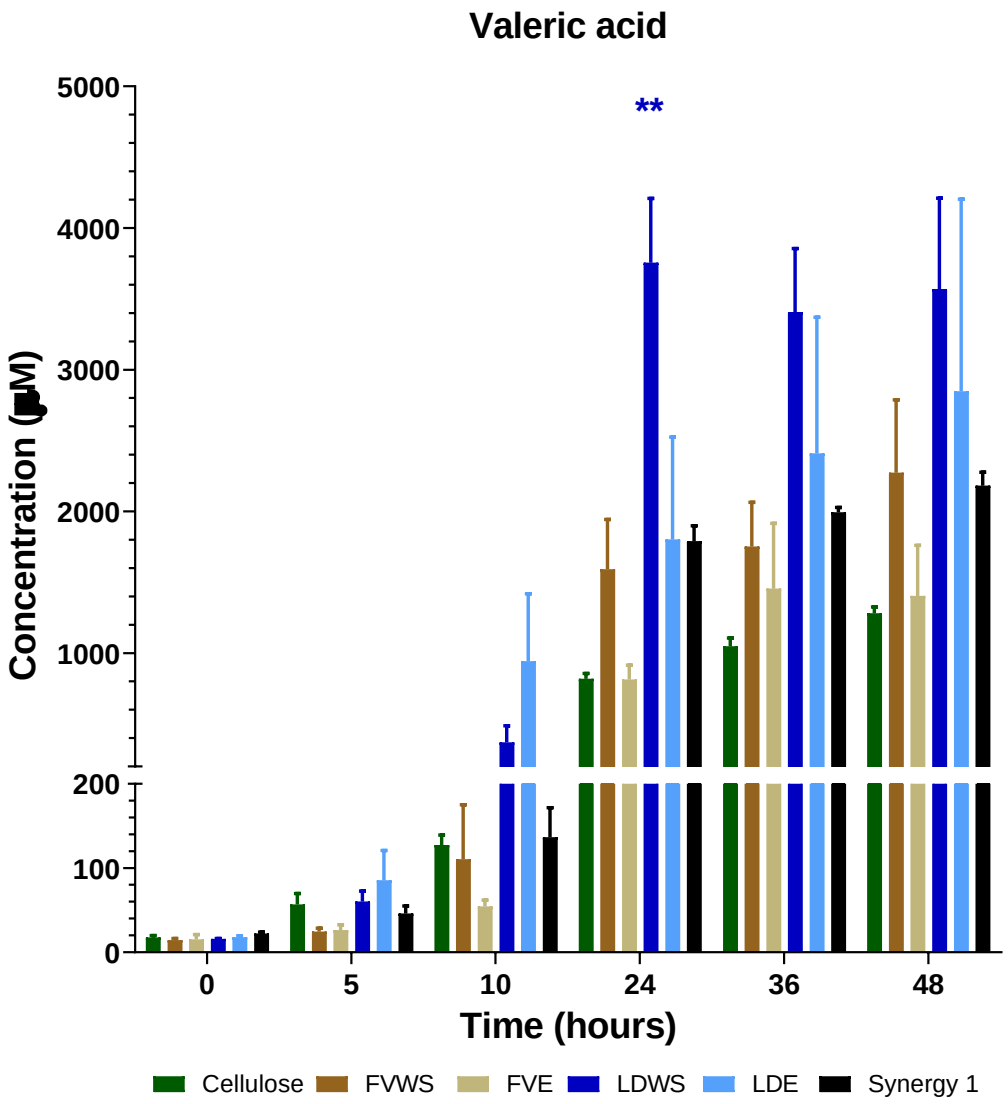

Figure 4-21. Valeric acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

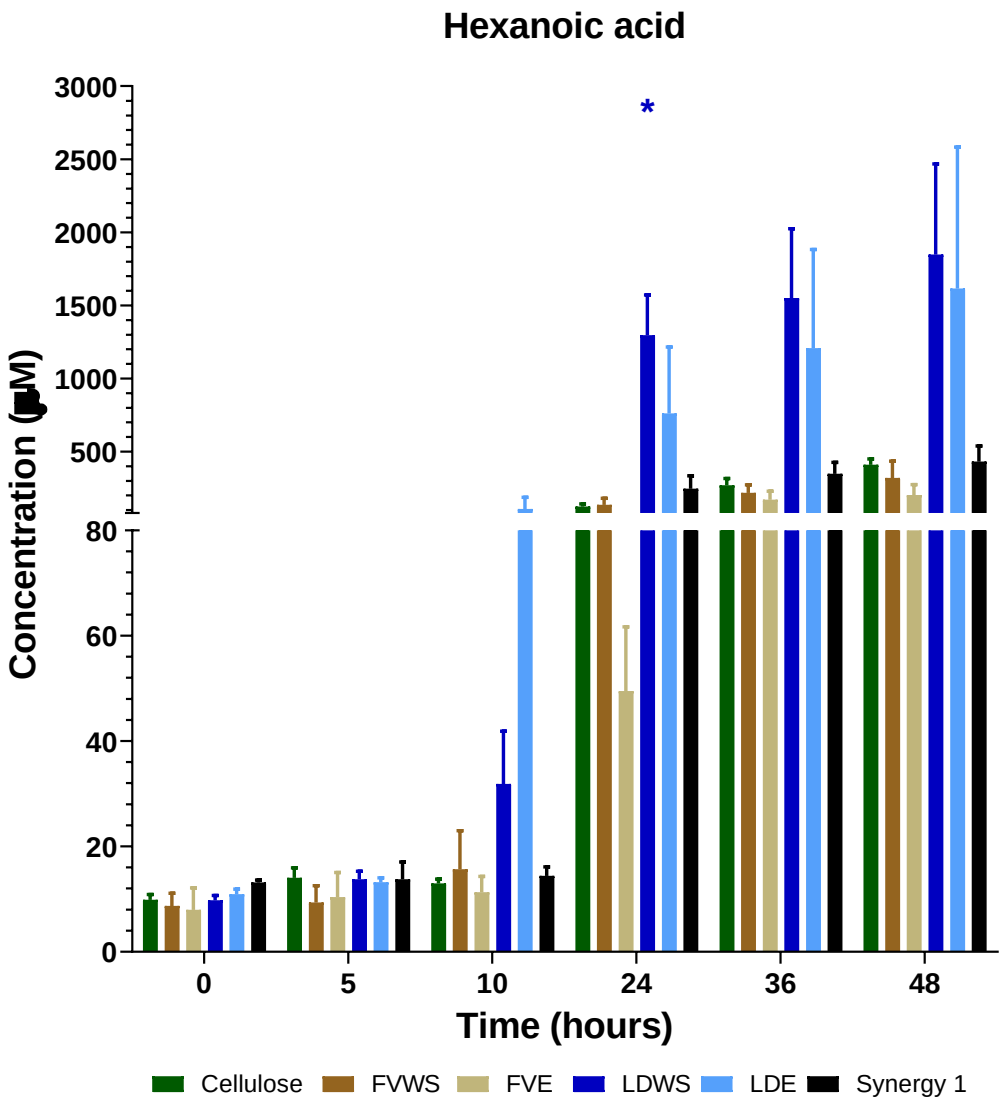

Figure 4-22. Haxanoic acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

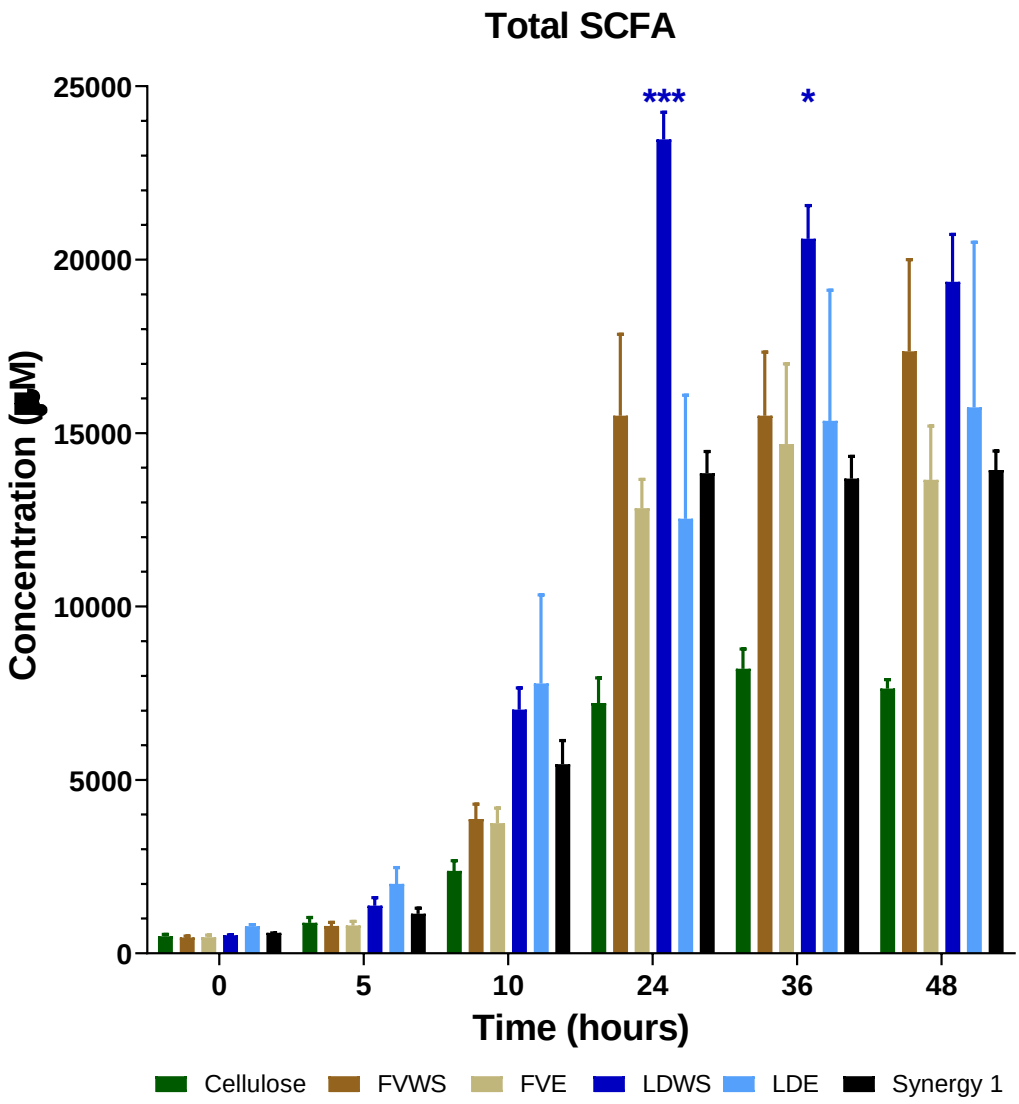

Figure 4-23 Total short-chain fatty acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

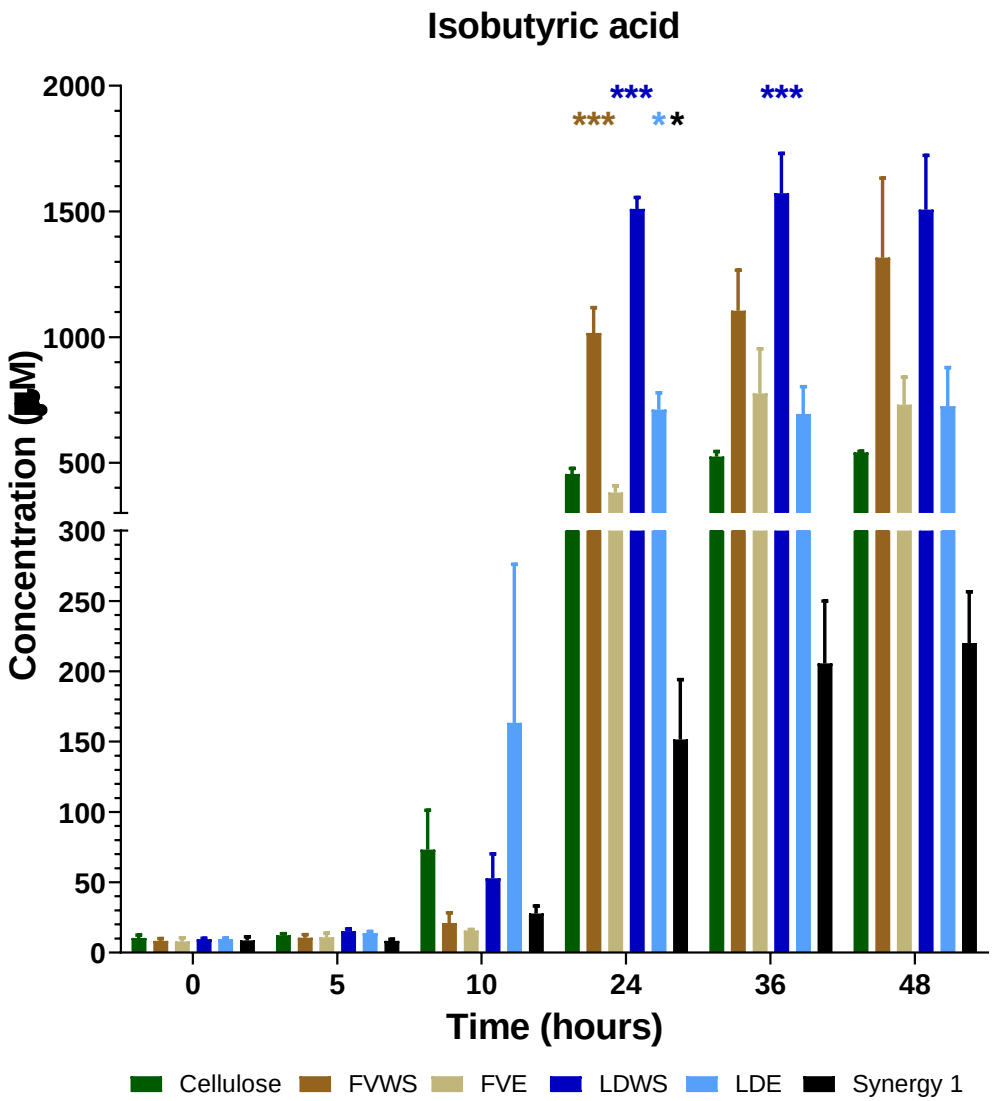

Figure 4-24. Isobutyric acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

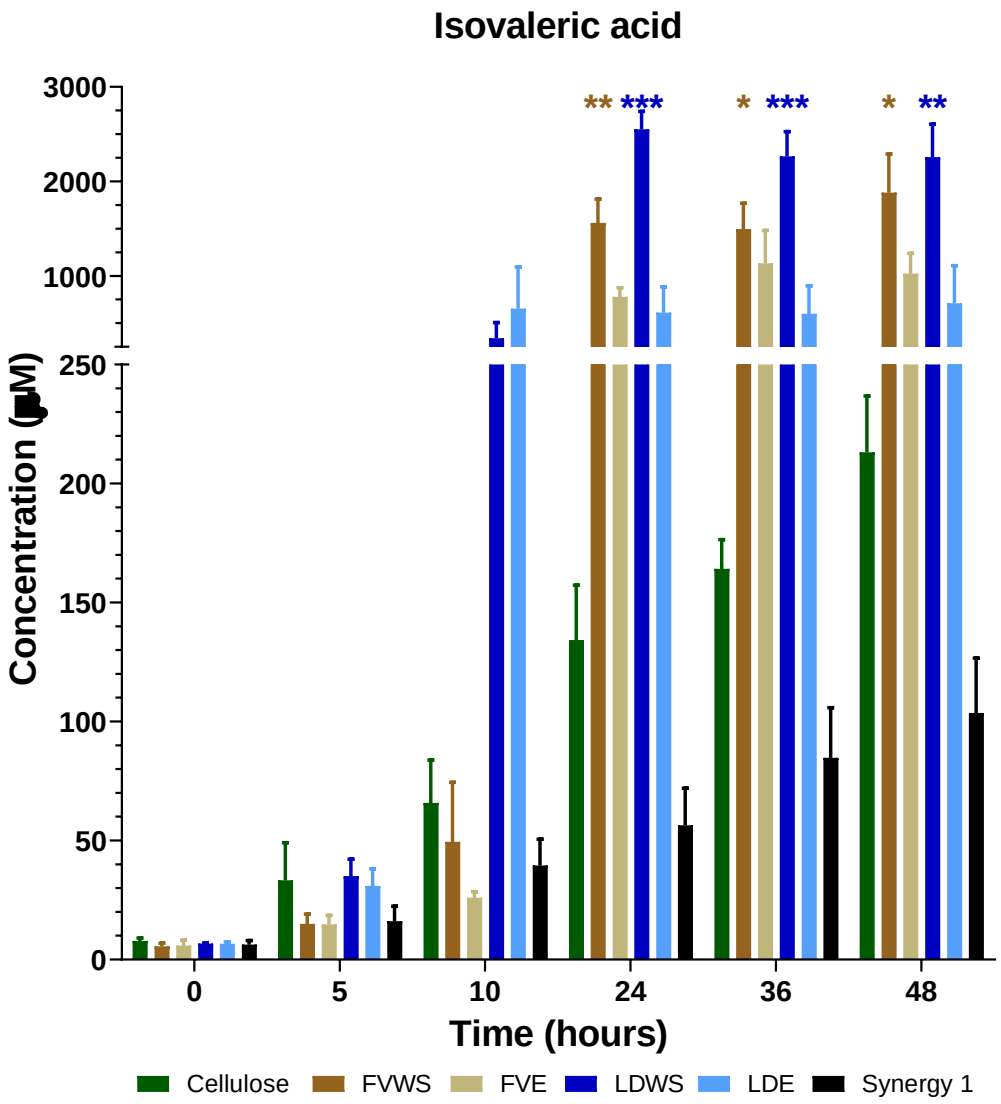

Figure 4-25. Isovaleric acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

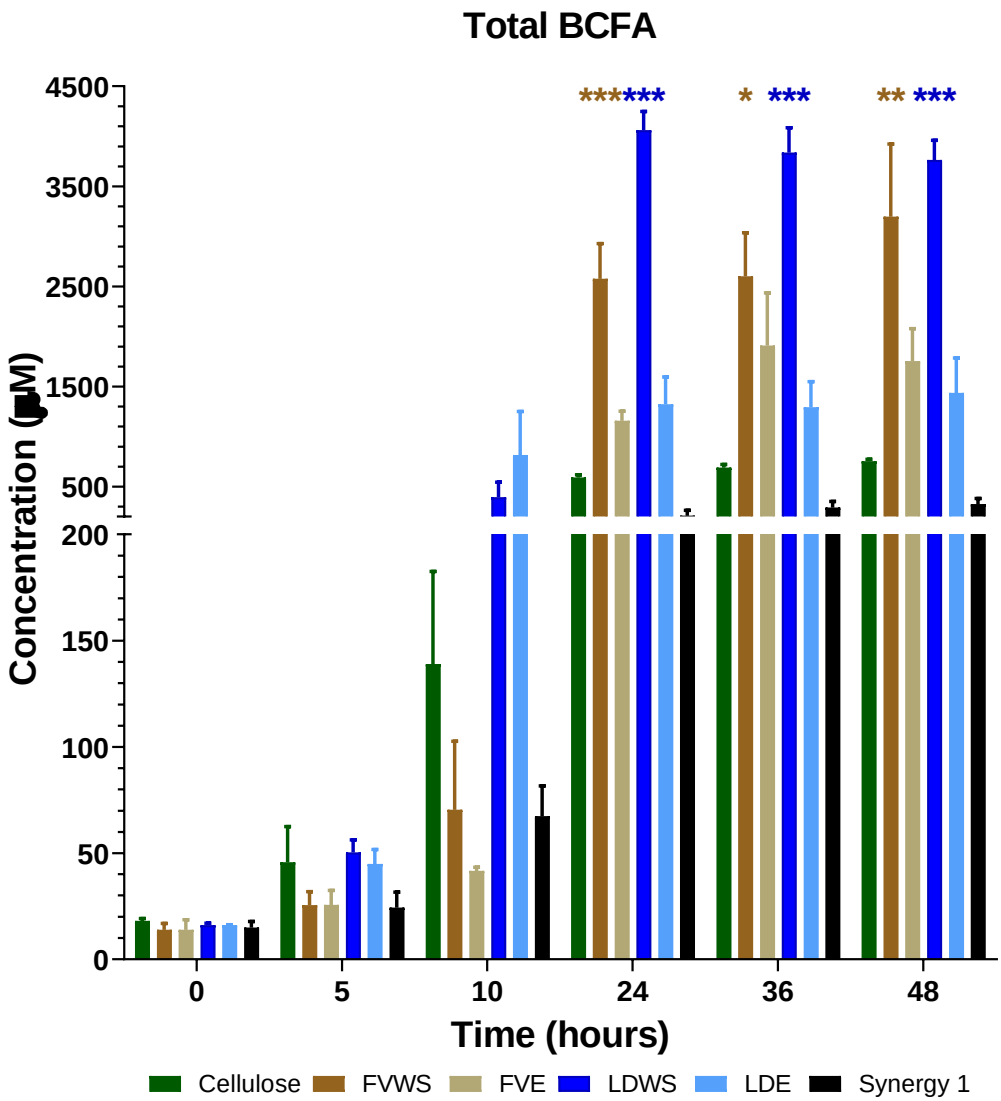

Figure 4-26. Total branched-chain fatty acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *F. vesiculosus* whole seaweed (FVWS), *F. vesiculosus* polysaccharide extract (FVE), *L. digitata* whole seaweed (LDWS), *L. digitata* polysaccharide extract (LDE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

4.9 Tables

Table 4-1. Mineral and trace element contents of the *Fucus vesiculosus* whole seaweed (FVWS) (n = 1) and *Fucus vesiculosus* polysaccharide rich extract (FVE) batch culture fermentation substrates. ppm – parts per million; RNI - Reference Nutrient Intake.

Fucus vesiculosus	**Whole Seaweed**					**Polysaccharide Rich Extract**				
Mineral / Trace Element	**Concentration (ppm)**	**Equivalent in mg/g**	**Amount (mg) in 5g**	**Amount (mg) in 10g**	**% RNI (mg/day)**	**Concentration (ppm)**	**Equivalent in mg/g**	**Amount (mg) in 5g**	**Amount (mg) in 10g**	**% RNI (mg/day)**
Aluminium	40.1	0.0401	0.201	0.401	Not applicable	29.2	0.0292	0.146	0.292	Not applicable
Chloride	46842	46.842	234.21	468.42	18.74	1630	1.63	8.15	16.3	0.652
Copper	<0.5	<0.0005	<0.005	<0.005	<0.0002	5.12	0.00512	0.0256	0.0512	4.27
Mercury	0.034	0.000034	0.00017	0.00034	Not applicable	<0.001	Negligible	Negligible	Negligible	Not applicable
Lead	0.847	0.000847	0.00424	0.00847	Not applicable	0.237	0.000237	0.00119	0.00237	Not applicable
Arsenic	43.83	0.04383	0.220	0.4383	Not applicable	63.47	0.0635	0.317	0.635	Not applicable
Cadmium	0.988	0.000988	0.00494	0.00988	Not applicable	0.187	0.000187	0.000935	0.00187	Not applicable
Iodine	219.8	0.220	1.099	2.20	1465.33	90	0.09	0.45	0.9	600
Sodium	24380	24.38	121.9	243.8	15.24	7941	7.941	39.71	79.41	4.96
Zinc	33.9	0.0339	0.170	0.339	3.57	19.5	0.0195	0.0975	0.195	2.05

Table 4-2. Mineral and trace element contents of the *Laminaria digitata* whole seaweed (LDWS) (n = 1) and *Laminaria digitata* polysaccharide rich extract (LDE) (n = 1) batch culture fermentation substrates. ppm – parts per million; RNI - Reference Nutrient Intake.

Laminaria digitata	**Whole Seaweed**					**Polysaccharide Rich Extract**				
Mineral / Trace Element	**Concentration (ppm)**	**Equivalent in mg/g**	**Amount (mg) in 5g**	**Amount (mg) in 10g**	**% RNI (mg/day)**	**Concentration (ppm)**	**Equivalent in mg/g**	**Amount (mg) in 5g**	**Amount (mg) in 10g**	**% RNI (mg/day)**
Aluminium	3.66	0.00366	0.0183	0.0366	Not applicable	16.5	0.0165	0.0825	0.165	Not applicable
Chloride	93785	93.79	468.93	937.85	37.514	810	0.81	4.05	8.1	0.324
Copper	<0.5	<0.0005	<0.005	<0.005	<0.0002	3.66	0.00366	0.0183	0.0366	3.05
Mercury	0.021	0.000021	0.000105	0.00021	Not applicable	<0.001	Negligible	Negligible	Negligible	Not applicable
Lead	0.076	0.000076	0.00038	0.00076	Not applicable	0.129	0.000129	0.000645	0.00129	Not applicable
Arsenic	71.875	0.0719	0.360	0.719	Not applicable	57.3	0.0573	0.287	0.573	Not applicable
Cadmium	0.069	0.000069	0.000345	0.00069	Not applicable	0.058	0.000058	0.00029	0.00058	Not applicable
Iodine	163.1	0.163	0.816	1.631	1087.33	130	0.13	0.65	1.3	866.67
Sodium	35160	35.16	175.8	351.6	21.975	5339	5.339	26.71	53.39	3.34
Zinc	29.9	0.0299	0.150	0.299	3.15	26.4	0.0264	0.132	0.264	2.78

Table 4-3. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *F. vesiculosus* whole seaweed (FVWS) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		FVWS		Effect	P-value
		Mean (%)	SD	Mean (%)	SD		
	Phylum						
10 h	Proteobacteria	4.083	0.104	6.853	0.395	↑	0.004
24 h	Planctomycetes	< 0.001	< 0.001	0.563	0.076	↑	< 0.0001
	Firmicutes	8.740	0.121	6.377	0.674	↓	0.007
	Family						
10 h	*Sva10996 marine group*	< 0.001	< 0.001	1.274	0.252	↑	0.000426
	Planctomycetaceae	2.159	0.113	1.681	0.073	↑	< 0.0001
	Peptostreptococcaceae	< 0.001	< 0.001	2.310	0.697	↓	< 0.0001
	Pasteurellaceae	< 0.001	< 0.001	1.753	0.916	↓	0.0377
	Granulosicoccaceae	0.038	0.066	1.035	0.283	↑	< 0.0001
	Flavobacteriaceae	2.426	0.180	1.647	0.054	↑	< 0.0001
24 h	*Veillonellaceae*	0.067	0.060	4.638	1.748	↓	0.0413
	Planctomycetaceae	< 0.001	< 0.001	1.676	0.203	↑	0.000833

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-3 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *F. vesiculosus* whole seaweed (FVWS) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		FVWS		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
	Genus						
10 h	*Terrisporobacter*	1.505	0.134	0.311	0.052	↓	< 0.0001
	Ruminococcus 1	0.586	0.032	0.291	0.045	↓	0.012
	Ruminiclostridium	0.707	0.100	0.375	0.070	↓	0.040
	Peptoclostridium	8.856	0.976	0.773	0.098	↓	< 0.0001
	Lachnospiraceae FCS020 group	0.313	0.107	0.081	0.070	↓	0.033
	Klebsiella	1.448	0.362	3.837	0.600	↑	0.050
	Haemophilus	0.885	0.067	0.193	0.168	↓	0.034
	Granulosicoccus	< 0.001	< 0.001	0.770	0.288	↑	< 0.0001
	Blastopirellula	< 0.001	< 0.001	1.110	0.165	↑	< 0.0001
24 h	*Ruminococcaceae UCG003*	0.884	0.434	0.139	0.133	↓	0.034
	Pseudoflavonifractor	0.517	0.174	< 0.001	< 0.001	↓	0.044
	Flavonifractor	0.691	0.191	0.125	0.049	↓	0.045
	Eubacterium hallii group	0.640	0.215	0.300	0.174	↓	0.040
	Blastopirellula	< 0.001	< 0.001	0.828	0.419	↑	0.001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-4. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *F. vesiculosus* polysaccharide extract (FVE) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		FVE		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
	Phylum						
10 h	Firmicutes	8.740	0.121	6.740	1.000	↓	0.022
	Family						
10 h	*Veillonellaceae*	15.323	2.295	7.471	0.190	↓	0.0181
	Ruminococcaceae	3.969	0.150	7.015	1.940	↑	0.0396
	Rikenellaceae	1.706	0.205	2.863	0.370	↑	0.0153
	Peptostreptococcaceae	4.264	0.629	6.454	10.404	↑	< 0.0001
	Christensenellaceae	0.703	0.070	1.453	0.112	↑	0.0197
24 h	*Veillonellaceae*	16.261	0.236	12.177	0.850	↓	0.0420

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose.

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-4 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *F. vesiculosus* polysaccharide extract (FVE) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		FVE		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
	Genus						
10 h	*Alistipes*	1.134	0.138	1.704	0.148	↑	0.039
	Terrisporobacter	1.505	0.134	0.443	0.081	↓	< 0.0001
	Ruminococcaceae UCG005	0.371	0.088	0.766	0.114	↑	0.018
	Pseudobutyrivibrio	0.675	0.156	1.654	0.231	↑	0.001
	Prevotella 7	0.369	0.143	1.544	0.706	↑	0.010
	Peptoclostridium	8.856	0.976	0.570	0.030	↓	< 0.0001
	Oscillospira	< 0.001	< 0.001	0.241	0.061	↑	0.021
	Megasphaera	9.518	1.665	2.549	0.223	↓	0.005
	Lachnospiraceae UCG001	< 0.001	< 0.001	0.322	0.082	↑	0.000106
	Lachnospiraceae NK4A136 group	0.218	0.033	0.602	0.096	↑	0.014
	Lachnospiraceae NC2004 group	0.033	0.056	0.389	0.159	↑	0.007
	Eubacterium ventriosum group	0.089	0.085	0.297	0.054	↑	0.035
	Eubacterium ruminantium group	< 0.001	< 0.001	0.268	0.094	↑	0.001
	Eubacterium coprostanoligenes group	0.119	0.109	1.347	0.558	↑	0.002
	Coprococcus 2	0.078	0.135	0.746	0.013	↑	0.005
	Christensenellaceae R7 group	0.436	0.018	0.866	0.056	↑	0.027
	Alistipes	1.134	0.138	1.704	0.148	↑	0.0389

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose.

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-4 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *F. vesiculosus* polysaccharide extract (FVE) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		FVE		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
Genus							
24 h	*Ruminococcaceae UCG003*	0.884	0.434	0.060	0.105	↓	0.018
	Megasphaera	10.625	0.288	5.147	2.515	↓	0.007
	Eubacterium hallii group	0.640	0.215	0.297	0.057	↑	0.038

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose.

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-5. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *L. digitata* whole seaweed (LDWS) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		LDWS		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
	Phylum						
10 h	Firmicutes	8.740	0.121	6.940	0.489	↓	0.0428
	Family						
10 h	*Unclassified Fucus*	0.067	0.060	5.321	0.169	↑	< 0.0001
	Unclassified Ectocarpus	< 0.001	< 0.001	4.078	0.694	↑	< 0.0001
	Peptostreptococcaceae	13.508	1.446	0.925	0.466	↓	< 0.0001
24 h	*Unclassified Fucus*	0.151	0.183	2.457	1.150	↑	0.0432
	Unclassified Ectocarpus	0.075	0.130	1.801	0.759	↑	0.000519

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-5 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *L. digitata* whole seaweed (LDWS) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		LDWS		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
Genus							
10 h	*Coprococcus 2*	0.078	0.135	0.584	0.209	↑	0.032
	Eubacterium hallii group	0.568	0.055	0.090	0.156	↓	0.006
	Eubacterium ventriosum	0.089	0.085	0.302	0.077	↑	0.030
	Lachnospiraceae NK4A136	0.218	0.033	0.565	0.136	↑	0.028
	Peptoclostridium	8.856	0.976	0.562	0.318	↓	< 0.0001
	Unclassified Ectocarpus	< 0.001	< 0.001	2.597	0.413	↑	< 0.0001
	Unclassified Fucus	0.045	0.040	3.393	0.032	↑	< 0.0001
	Terrisporobacter	1.505	0.134	0.142	0.130	↓	< 0.0001
24 h	*Unclassified Fucus*	0.104	0.126	1.655	0.723	↑	0.042
	Unclassified Ectocarpus	0.052	0.090	1.214	0.474	↑	0.000306
	Ruminococcaceae UCG004	< 0.001	< 0.001	0.056	0.050	↑	0.049
	Ruminococcaceae UCG003	0.884	0.434	0.180	0.158	↓	0.048
	Parasutterella	0.606	0.069	0.909	0.081	↑	0.043
	Eubacterium hallii group	0.640	0.215	0.119	0.018	↓	0.002
	Coprococcus 2	0.052	0.091	0.382	0.205	↑	0.018
	Butyricimonas	0.234	0.058	0.577	0.005	↑	0.000294

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-6. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *L. digitata* polysaccharide extract (LDE) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		LDE		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
	Phylum						
No statistically significant differences observed							
	Family						
10 h	*Peptostreptococcaceae*	13.508	1.446	8.791	2.208	↓	0.00565
	Genus						
10 h	*Peptoclostridium*	8.856	0.976	5.639	1.172	↓	0.002
	Serratia	0.030	0.051	7.451	3.981	↑	0.001
	Terrisporobacter	1.505	0.134	1.030	0.188	↓	0.012
	Yersinia	0.099	0.047	4.127	0.720	↑	< 0.0001
24 h	*Yersinia*	0.089	0.154	0.571	0.348	↑	0.032
	Solobacterium	0.182	0.072	2.150	1.343	↑	0.016
	Serratia	0.081	0.141	4.460	3.525	↑	0.027
	Eubacterium hallii group	0.640	0.215	0.234	0.049	↓	0.013

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-7. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) Synergy 1 when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		Synergy 1		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
	Phylum						
10 h	Actinobacteria	0.723	0.080	1.700	0.369	↑	0.012
24 h	Actinobacteria	0.377	0.080	2.123	0.137	↑	< 0.0001
	Family						
10 h	*Streptococcaceae*	1.116	0.089	5.697	0.892	↑	< 0.0001
	Peptostreptococcaceae	13.508	1.446	6.086	1.414	↓	0.000104
	Enterococcaceae	3.896	0.510	14.013	2.035	↑	0.027297
	Clostridiaceae 1	3.356	1.180	9.621	4.907	↑	0.038454
	Bifidobacteriaceae	1.837	0.320	4.543	0.940	↑	0.003847
24 h	*Streptococcaceae*	0.448	0.108	5.200	0.522	↑	< 0.0001
	Erysipelotrichaceae	1.599	0.469	4.879	1.185	↑	0.018202
	Coriobacteriaceae	0.566	0.184	1.645	0.108	↑	0.000126
	Bifidobacteriaceae	1.069	0.194	6.174	0.389	↑	< 0.0001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-7 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) Synergy 1 when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		Synergy 1		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
Genus							
10 h	*Terrisporobacter*	1.505	0.134	0.778	0.186	↓	< 0.0001
	Streptococcus	0.742	0.061	4.175	0.713	↑	< 0.0001
	Peptoclostridium	8.856	0.976	4.385	1.000	↓	< 0.0001
	Erysipelotrichaceae UCG003	0.474	0.026	0.229	0.045	↓	0.036
	Enterococcus	2.589	0.333	10.240	1.327	↑	0.007
	Clostridium sensu stricto 1	2.230	0.781	7.077	3.711	↑	0.032
	Catenibacterium	1.302	0.210	3.197	0.718	↑	0.019
	Bifidobacterium	1.221	0.216	3.318	0.644	↑	0.001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 4-78 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) Synergy 1 when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		Synergy 1		Effect	*P*-value
		Mean (%)	SD	Mean (%)	SD		
Genus							
24 h	*Veillonella*	2.596	0.280	8.125	0.817	↑	0.003
	Streptococcus	0.306	0.070	3.724	0.464	↑	< 0.0001
	Holdemanella	0.205	0.031	0.550	0.154	↑	0.037
	Eubacterium hallii group	0.640	0.215	0.223	0.053	↓	0.011
	Collinsella	0.219	0.060	1.038	0.070	↑	< 0.0001
	Citrobacter	2.009	0.282	0.939	0.100	↓	0.029
	Catenibacterium	0.974	0.280	3.366	0.783	↑	0.001
	Blautia	1.055	0.277	2.120	0.144	↑	0.020
	Bifidobacterium	0.730	0.125	4.417	0.340	↑	< 0.0001
	Anaerotruncus	0.757	0.207	0.157	0.047	↓	0.040

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Chapter 5

Experimental Chapter

5 Effects of the edible Irish red seaweed, *Palmaria palmata*, on the composition and metabolic activity of the human gut microbiota using an *in vitro* model of the distal colon

5.1 Abstract

Palmaria palmata (*P. palmata*), a red seaweed grown and harvested in Ireland, has been proposed as a source of prebiotic fibre due to the presence of xylan, however there is a paucity of evidence on its impact on the human microbiota. This study used an *in vitro* batch culture model of the distal colon to investigate the effect of *P. palmata* whole seaweed (PPWS) as well as a crude polysaccharide-rich extract from *P. palmata* (PPE) on gut microbiota composition (culture-dependent enumeration, qPCR, and 16S rRNA amplicon sequencing) and metabolism (short chain fatty acid quantification via GC-MS). Cellulose and Synergy 1 were used as negative and positive controls, respectively.

PPWS & PPE treatments had no impact on either alpha or beta measures of microbial diversity, however, both PPWS & PPE were shown to significantly stimulate *Bifidobacterium* populations. Both treatments also induced significant changes in a range of genera including *Bacteroides*, *Butyricimonas*, *Dorea*, *Parabacteroides*, *Phascolarctobacterium*, and *Pseudobutyrivibrio* (PPWS); *Butyricimonas*, *Coprococcus*, *Lachnospiraceae* UCG008, and *Prevotella* (PPE). No significant changes in any measured SCFA were noted when PPE was included in the fermentation, while inclusion of PPWS in the fermentation resulted in significantly higher concentrations of total SCFA (24 and 36 h, $p < 0.05$) and the individual SCFA, propionic acid (10 h, $p < 0.05$), butyric acid (24, 36 and 48 h, $p <$

0.05), valeric acid (10, 24, 36 and 48 h, $p < 0.05$), and hexanoic acid (10, 24, 36 and 48 h, $p < 0.05$) when compared to cellulose.

The results of this study indicate that the both PPE & PPWS can favourably impact microbiota composition, however the notable increase SCFA production induced by PPWS make it a more promising prebiotic candidate and warrants further investigation.

5.2 Introduction

Seaweed consumption is known to make a considerable contribution to the indigenous diet of Asian countries such as China and Japan, with a reported intake of 5.2g/day and 5.3g/day, respectively (Chen et al., 2018; Matsumura, 2001). Evidence, albeit limited, from observational studies indicates that seaweed consumption may be associated with a reduced risk of non-communicable diseases (NCDs), including cardiovascular disease, type two diabetes, and obesity (Brown et al., 2014). While seaweeds contain a range of health promoting nutrients associated with reduced NCD risk, there is an increasing interest in the potential health promoting impact of the high complex polysaccharide content in seaweeds (MacArtain et al., 2007). The health benefits of dietary fibre has been attributed to the physicochemical viscous properties affecting digestion and absorption in the upper gastrointestinal tract, as well as their uptake, utilisation and metabolism by the gut microbiota (Anderson et al., 2009; O'Grady et al., 2019). Furthermore, fibre-mediated modulation of the gut microbiota has the potential to exert prebiotic effects by enabling selective metabolism by gut commensals to improve host health (Gibson et al., 2017). Historically, the prebiotic potential of fibre was associated with increased numbers of bifidobacteria and/or lactobacilli, and the

increased production of health promoting SCFA such as propionate or butyrate. More recent evidence indicates that dietary fibre may improve health though the maintenance or enhancement of gut bacterial diversity to inhibit pathogens, to stimulate putative next generation probiotics (e.g. strains of *Bacteroides, Akkermansia* and *Faecalibacterium*) (O'Toole et al., 2017), and to protect against metabolic and inflammatory disease (Desai et al., 2016; Sonnenburg and Sonnenburg, 2014; Valdes et al., 2018).

The potential health benefits of prebiotics such as inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) include increased mineral bioavailability, pathogen inhibition, immune system stimulation, reduced blood lipids, improved insulin sensitivity, and improved brain function through the influence of bacterial metabolites on the microbiota-gut-brain axis (Burokas et al., 2017; Carlson et al., 2018; Gibson et al., 2017). Current interest in seaweeds as a potential source of prebiotic fibres has focused primarily on brown seaweed fibre components where polysaccharides such as alginate, laminarin and fucoidan have previously demonstrated increased production of acetate, propionate, butyrate, and total SCFA during *in vitro* fermentation experiments. Modulation of bacterial composition from these experiments has included an increase in populations such as *Bifidobacterium*, *Bacteroides*, *Lactobacillus*, *Roseburia*, *Parabacteroides*, *Parasutterella*, *Fusicatenibacter*, *Coprococcus*, *Faecalibacterium* (Charoensiddhi et al., 2017, 2016; Fu et al., 2018; Strain et al., 2019). There is emerging evidence for the prebiotic activity of sulphated galactans from red seaweeds (Bajury et al., 2017; Bhattacharya et al., 2015; Di et al., 2018; Hu et al., 2006; Ramnani et al., 2012;

Rodrigues et al., 2016), albeit limited evidence exists with regards to the effect of red seaweed xylan polysaccharides on gut microbial composition and metabolism.

There is increasing interest in the potential health benefits of the red seaweed *P. palmata* owing to its high xylan content, and further, its widespread consumption in European and coastal communities. The prebiotic potential of xylan has been reported for cereal derived 1,4- linked β-D-xylopyranose xylan, however, this structure differs to the 1,3- and 1,4- linked β-D-xylopyranose xylan present in *P. palmata* (Grote, 2017; Lahaye and Rochas, 1991).Using an *in vitro* batch culture model of the human colonic microbiota, this study aimed to evaluate the fermentability of *P. palmata* whole seaweed (PPWS) and a *P. palmata* polysaccharide rich fibre extract (PPE) in comparison to cellulose. Furthermore, to obtain indications on the prebiotic potential of PPWS and PPE, we investigated any effects on microbiota composition and SCFA production.

5.3 Materials and Methods

5.3.1 Chemicals and reagents

Chemicals were purchased from Sigma Aldrich (St. Louis, USA) unless otherwise stated. Reagents used during HPSEC and GC-MS were HPLC grade. Reagents used for DNA extraction, qPCR, and 16S rRNA amplicon sequencing were molecular biology grade.

5.3.2 Seaweed harvest

P. palmata was harvested from Spiddal, Co. Galway, Ireland (53° 14' 48" North, 9° 18' 10" West) in September 2014. Only the leaf was used, and the stipe was removed from the holdfast during harvesting. The seaweed was immediately

washed in seawater to remove contaminants (e.g. epiphytes, molluscs and other seaweeds). Washed seaweed was freeze-dried, ground into a fine powder using an electronic blender and stored at -20 °C until required.

5.3.3 Polysaccharide-rich extract (PPE) production

P. palmata powder was shaken in dH_2O (1:20, w/v) for two mins to reduce the initial salt content of the seaweed and then filtered through muslin cloth to remove water. A total of 30g of washed *P. palmata* whole seaweed (PPWS) was freeze-dried and stored for *in vitro* digestion and batch culture fermentation experiments. The seaweed powder underwent hot acid extraction, neutralisation, desalination, ethanol precipitation, and lyophilisation to generate a crude *P. palmata* polysaccharide-rich extract powder (PPE).

5.3.4 PPE characterisation

The average molecular weight of PPE was determined using a modified high performance size exclusion chromatography method (Gomez-Ordonez et al., 2012). In brief, separation was performed on a PL aquagel-oh mixed-H 8µm SEC analytical column (7.5 x 300 mm i.d) with isocratic elution at 50°C and a flow rate of 0.6 mL/min and a run time of 31 mins, using a 50mM ammonium formate mobile phase and a ten-point pullulan standard curve (0.34 to 708 kDa).

Total carbohydrate content of FVE and LDE was quantified using a modified phenol-sulfuric acid colorimetric assay (DuBois et al., 1956). Sulphate content of FVE and LDE was quantified using a modified Azure A colorimetric assay, expressed as percentage equivalents of purified fucoidan (Fucoidan from *Fucus vesiculosus* F5631, Sigma Aldrich, USA) (Torode et al., 2015). Structural information of FVE and

LDE was determined using Fourier Transform Infrared Spectroscopy (FTIR) using the Bruker Tensor 27 FT-IR spectrophotometer (Bruker Corporation, UK) with OPUS 5.5 software.

5.3.5 Mineral and trace element content of PPWS and PPE

Mineral and trace element analysis of PPWS and PPE (1 x 10g of each) was outsourced to Advanced Laboratories (Salt Lake City, Utah, USA). Concentrations of aluminium, copper, sodium and zinc were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Concentrations of mercury, lead, arsenic, and cadmium were determined by inductively coupled plasma mass spectrometry (ICP-MS). Iodine concentration was determined using AOAC 932.21.

5.3.6 *In vitro* digestion

An *in vitro* simulated digestion was completed on FVWS, LDWS, FVE and LDE using a standardised method (Minekus et al., 2014) with oral, gastric, and intestinal phases of digestion. All simulated fluids were incubated at 37°C before use and at all digestion stages the fluids were incubated at 37°C in an orbital shaker (160 rpm). The oral suspension was incubated for 2 mins, while both the gastric and intestinal suspensions were incubated for 2 hrs. The intestinal phase digesta was then dialysed for 24 hrs using 1kDa dialysis tubing (Spectrum Labs, USA) and the retentate was freeze dried (Labconco, USA) to obtain powders prior to batch culture fermentation.

5.3.7 *In vitro* batch culture fermentation

A 20% faecal slurry was prepared following the methods of O'Donnell *et al.* (O'Donnell et al., 2016). The resulting faecal bacteria suspension was amended

with sterile glycerol to a final concentration of 25% (v/v) and stored frozen at -80°C until use. Nutrient basal medium stock solution was prepared using the methodology of Fooks and Gibson (Fooks and Gibson, 2003). *In vitro* batch culture fermentation was performed using the Multifors parallel bioreactor with Iris 6.0 software (Infors HT, Basel, Switzerland). A total of 2g of either PPE, PPWS, cellulose (negative control), or Synergy 1 (positive control) was added to 190ml nutrient basal medium stock solution (final carbohydrate concentration = 1% w/v). Vessels were sparged with N_2 gas for 2 hrs (2 psi) before inoculation with 10ml of 20% faecal slurry (previously thawed at 37°C). Vessels were automatically stirred at 200 revolutions min^{-1}, maintained at pH 6.8 (dropwise addition of 1M HCl or 1M NaOH), and incubated at 37°C. A 7mL sample was taken at t = 0, 5, 10, 24, 36 and 48 hrs for culture-dependent, culture-independent (qPCR and 16S rRNA amplicon sequencing), and SCFA analysis. Substrate fermentation was performed in triplicate.

5.3.8 Culture-dependent analysis

100 µL of fermentation sample was serially diluted in a maximum recovery diluent (10^1 - 10^5) and dilutions were plated in triplicate on modified De Man, Rogosa and Sharpe agar (MRS) agar selective for *Bifidobacterium* (sterile filtered with 5ml/100ml Mupirocin and 1ml/100ml Nystatin), and *Lactobacillus* selection agar (LBS) selective for *Lactobacillus* (sterile filtered with 1ml/100ml Nystatin). Cultures were incubated anaerobically at 37°C for 72 hrs before counting. Counts were corrected for each dilution factor and then calculated as $\log_{10}$ CFU/mL.

5.3.10 DNA extraction

Genomic DNA was extracted from samples after 0, 5, 10, 24, 36, and 48 hrs fermentation using the PowerFecal DNA extraction kit (Mo Bio Laboratories, USA) according to manufactures' instructions. The bead beating step was completed using the Mo Bio vortex adapter.

5.3.11 qPCR

Total bacteria, *Lactobacillus*, and *Bifidobacterium* were quantified using qPCR. Primer sequences used for qPCR (Target: Forward primer 5'-3'; Reverse primer 5'-3'; Size bp; Tm °C): Total Bacteria (Eubacterial): ACTCCTACGGGAGGCAGCAG; ATTACCGCGGCTGCTGG; 200 bp; 60°C. *Lactobacillus* genus: GCAGCAGTAGGGAATCTTCCA; GCATTYCACCGCTACACATG; 349 bp; 60°C. *Bifidobacterium* genus: CTCCTGGAAACGGGTGGT; GCTGCCTCCCGTAGGAGT; 203 bp; 60°C. A standard curve of 10^9-10^3 CFU/ml was prepared in duplicate for each plate. A PCR master mix was prepared with the forward and reverse primers, SYBR® FAST pPCR Master Mix (KAPA Biosystems, USA), and PCR water. 1 µl of sample DNA or PCR water (negative control) was added to 9 µl master mix per well (reaction volume = 10 µl) and ran in duplicate on two plates (n = 4). The Lightcycler® 480 Instrument II (Roche, Switzerland) was used with the following PCR conditions: denaturation = 1 cycle; amplification = 40 cycles; melting = 1 cycle; cooling = 1 cycle. Target temperature was 95°C with a hold time of 3 min and a ramp rate of 4.4°C/sec.

5.3.13 16S rRNA amplicon sequencing

Illumina MiSeq sequencing library preparation was completed following the 16S metagenomic sequencing library protocol (Illumina, USA) and the methods of Fouhy *et al*. (Fouhy et al., 2015). Amplicon PCR: Genomic DNA was amplified using primers specific to the V3-V4 hypervariable region of the 16S ribosomal RNA gene to create a 460bp amplicon. These primers also incorporated the Illumina overhang adaptor (Forward primer 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

reverse primer 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Each PCR reaction contained 2.5 µL template DNA, 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 12.5 µL 2X Kapa HiFi Hotstart ready mix (KAPA Biosystems, USA) - 25µL final reaction volume. PCR amplification was carried out using the Applied Biosystems 2720 thermal cycler (Life Technologies, USA) with the following parameters: heated lid 110°, 95°C for 3 mins; then 25 cycles of: 95°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs; hold at 72°C for 5 mins; hold at 4°C. Successful PCR products were cleaned using Agentcourt AMPure XP kit (Beckman Coulter Genomics, UK) a second PCR reaction was completed to attach Illumina sequencing adapters onto the amplicons, using the Nextera XT Index kit (Illumina, USA). The DNA concentration of each sample was determined using the Qubit High Sensitivity DNA kit and the Qubit 3 Fluorometer (Invitrogen, USA). DNA samples were then pooled as an equimolar mix and sequenced on the MiSeq sequencing platform at Teagasc, Moorepark, Ireland following standard Illumina sequencing protocols for the 2 × 250 cycle V3 Kit.

5.3.14 Bioinformatics

Two hundred and fifty base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (Caporaso et al., 2010). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123.

5.3.15 Short chain fatty acid and branched chain fatty acid analysis

GC-MS analysis was carried out using a modified protocol described by Garcia-Villalba *et al.* (Garcia-Villalba et al., 2012). In brief, phosphoric acid was added to samples to a final concentration of 0.5% (v/v) prior to ethyl acetate extraction (1:1 v/v). 180µl organic phase was added to a GC vial alongside 20µl of 4-methyl valerate internal standard. A standard curve of 10, 20, 50, 100, 500, 1000, 5000, 10000, 50000, and 100,000 µM SCFA mix containing acetic acid, propionic acid, n-butyric acid, i-butyric acid, valeric acid, i-valeric acid, and hexanoic acid was ran within every sample batch. Quality control consisted of two 50 µM and two 100 µM standard mixes every sixteen vials and ethyl acetate blanks every six vials, and between each standard vial/QC to prevent carryover. The GC-MS system consisted of an Agilent 6890N (Agilent Technologies, USA), equipped with an Agilent 7683 AutoSampler and 7683B injector, coupled to an Agilent 5973 inert mass selective

detector. Agilent MassHunter GC/MS Acquisition software was used. The GC was fitted with a DB-WAXetr capillary column (30m length, 0.25mm i.d, 0.25µm film thickness), with helium used as the carrier gas (1.2mL/min). Injections were made in splitless mode with an injection volume of 1µL (10 µL syringe) and an injection temperature of 250°C. The syringe undertook four pre-washes and four post-washes in hexane. The initial column temperature was 90°C and ramped to 150°C at 15°C/min, then to 170°C at 5°C/min, then to 230°C at 20°C/min, where it was maintained for 2 mins. Total run time was 14 mins. Solvent delay was 2.5 mins. The detector was operated in electron impact ionisation mode.

5.3.16 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 24) and GraphPad Prism 8 software. Graphs were made in GraphPad Prism 8 software. To determine the significant effects of each treatment on bacterial enumeration (culture and qPCR), a non-parametric Kruskal-Wallis test with a Bonferroni correction for multiple comparisons and a significance level of 0.05 was used to compare each treatment to cellulose. To determine the significant effects of each treatment on SCFA concentrations, statistical significance was determined between each treatment and cellulose using a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons with a significance level of $p \leq 0.05$.

Statistical analysis of 16S rRNA amplicon sequencing data was carried out using Calypso online software (version 8.68) (Zakrzewski et al., 2017). Data were normalized using cumulative sum scaling and $\log_2$ transformed to account for the non-normal distribution of sequencing data (Paulson et al., 2013). Up to 20,000

taxa with > 0.01% abundance were used in the analysis. Chloroplasts and cyanobacteria were removed from the analysis.

Alpha diversity was determined using rarefied Chao1, Evenness, Shannon and Simpson indices. Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). Beta diversity was determined Bray-Curtis dissimilarity distance matrices for each fermentation substrate and cellulose at a given time point. A Permutational multivariate analysis of variance (PerMANOVA) was used to determine the statistical difference between Bray–Curtis dissimilarity indices of beta diversity.

Statistical significance of mean bacterial relative abundances, when compared to cellulose, at the phylum, family, and genus level after 0, 10, and 24 hrs fermentation was determined using a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons and a significance level of $p \leq 0.05$. Discriminate taxa between fermentation substrates and cellulose at a given time point were identified using linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011).

5.4 Results

5.4.1 PPE characterisation

The average molecular weight of PPE was 4.5 kDa and the molecular weight distribution ranged from 1 kDa to 10 kDa. The total carbohydrate content of PPE was 93.9 ± 4.64 % while the total sulphate content, expressed as fucoidan equivalents, was negligible (below the limit of detection of the

spectrophotometer). Qualitative determination of monosaccharide content (**Figure 5-1**) indicated that hydrolysed PPE consisted of xylose. **Figure 5-2** shows the FTIR spectra of PPE pre- and post- *in vitro* digestion. The main band at 1038cm^{-1} could be attributed to the C-C ring and C-O (C-OH side chain) stretching vibrations of 1, 4-linked xylan (Buslov et al., 2009; Kačuráková et al., 1999). Several major overlapping bands from 1168 cm^{-1} (C-C, C-O, or C-O-C stretch) to 1076 cm^{-1} (C-C or C-O stretch) and 991 cm^{-1} (C-H_3 or C-OH bend) could indicate a large proportion of sugar rings present in the PPE sample. Absorption at 899cm^{-1} indicates a C-1-H bend within the pentose sugar (Kačuráková et al., 1999). Similar absorption bands were produced between pre- and post-digested samples, with differences in water absorption at 1628cm^{-1}.

5.4.2 Mineral and trace element analysis

Table 5-1 shows data from the mineral and trace element analysis of PPE and PPWS fermentation substrates. The iodine content of PPE and PPWS was 0.15 mg/g and 0.52 mg/g, respectively.

5.4.3 Batch culture fermentation

5.4.3.1 Bacterial enumeration: culture-dependent analysis

There was no statistical difference in the number of cultured *Lactobacillus* spp. and *Bifidobacterium* spp. colonies when either PPWS, PPE, or Synergy 1 were included in the fermentation when compared to cellulose at any time point after false discovery rate correction for multiple comparisons (**Figure 5-3**).

5.4.3.3 Bacterial enumeration: qPCR

The total bacteria present in vessels fermented with either PPWS, PPE, or Synergy 1 was not significantly different to vessels fermented with cellulose at any time point (**Figure 5-4**). Inclusion of PPWS in the fermentation resulted in a significant reduction of *Lactobacillus* spp. after 5 hrs (p = 0.0046, q = 0.007) and 10 hrs (p = 0.0034, q = 0.0103) when compared to cellulose, but not thereafter (**Figure 5-5**). Vessels treated with PPWS had significantly lower concentrations of *Bifidobacterium* spp. when compared to cellulose at 0 hrs (p = 0.001, q = 0.0029) and 5 hrs (p = 0.0083, q = 0.0124) fermentation, however, counts of *Bifidobacterium* spp. were significantly higher in vessels fermented with PPWS when compared to cellulose after 24 hrs (p = 0.0224, q = 0.0224) (**Figure 5-6**). PPE fermentation resulted in a significant reduction in *Bifidobacterium* spp. when compared to cellulose after 5 hrs (p = 0.017, q = 0.005), however, *Bifidobacterium* spp. were significantly higher in vessels fermented with PPE compared to cellulose after 10 hrs (p = 0.0283, q = 0.0424), 24 hrs (p = 0.0065, q = 0.0098), 36 hrs (p = 0.0029, q = 0.0043) and 48 hrs (p = 0.0018, q = 0.0028) (**Figure 5-6**). *Lactobacillus* spp. were significantly lower after 5 hrs PPE fermentation when compared to cellulose (p = 0.0025, q = 0.007) but were not significantly different thereafter (**Figure 5-5**). Synergy 1 fermentation resulted in significantly higher *Bifidobacterium* spp. when compared to cellulose after 10 hrs (p = 0.0156, q = 0.0424), 24 hrs, 36 hrs, and 48 hrs fermentation (p < 0.0001, q < 0.0001) (**Figure 5-6**).

5.4.3.5 16S rRNA amplicon sequencing: effect of PPWS and PPE fermentation on microbial diversity

There were no significant differences between any fermentation substrates when compared to cellulose for the alpha diversity indices of Chao1, Evenness, Simpson Index and Shannon Index (**Figure 5-7**), nor the Bray-Curtis dissimilarity index of beta diversity at 0, 10, or 24 hrs fermentation ($p > 0.5$).

5.4.3.6 16S rRNA amplicon sequencing: effect of PPE and PPWS fermentation on microbial abundance

Relative abundances of the bacteria present in vessels fermented with cellulose, PPWS, PPE, and synergy 1 are shown at the phylum level (**Figure 5-8**), family level (**Figure 5-9**), and genus level (**Figure 5-10**, **Figure 5-11**, and **Figure 5-12**).

The mean relative abundance of the genus *Alloprevotella* was significantly higher in vessels treated with PPWS treatment when compared to cellulose after 10 hrs (0.458 ± 0.066 % vs < 0.001 %, $p = 0.005$). PPWS treatment significantly reduced the relative abundance of several genera when compared to cellulose after 10 hrs, including *Tyzzerella* 3 (< 0.001 % vs 0.435 ± 0.186 %, $p = 0.006$), *Lachnospiraceae* FCS020 group (0.608 ± 0.108 % vs 0.862 ± 0.091 %, $p = 0.030$), *Faecalibacterium* (1.211 ± 0.127 % vs 1.519 ± 0.060 %, $p = 0.021$), *Erysipelotrichaceae* UCG003 (0.655 ± 0.099 % vs 0.996 ± 0.059 %, $p = 0.003$), and *Christensenellaceae* R7 group (0.810 ± 0.081 % vs 1.030 ± 0.082 %, $p = 0.028$) (**Table 5-2**).

After 24 hrs fermentation in the presence of PPWS, the relative abundance of four genera were significantly higher when compared to cellulose: *Pseudobutyrivibrio* (1.688 ± 0.235 % vs 1.046 ± 0.140 %, $p = 0.021$), *Butyricimonas* (1.448 ± 0.212 % vs

0.881 ± 0.0125 %, *p* = 0.021), *Bifidobacterium* (1.955 ± 0.184 % vs 1.221 ± 0.082 %, *p* = 0.002), and *Bacteroides* (3.365 ± 0.286 % 2.629 ± 0.061 %, *p* = 0.007). After 24 hrs PPWS fermentation, the relative abundance of three bacterial genera were significantly lower when compared to cellulose: *Ruminococcaceae* UCG002 (< 0.001 % vs 1.146 ± 0.131 %, *p* = 0.006), *Clostridium* sensu stricto 1 (0.687 ± 0.610 % vs 1.823 ± 0.308 %, *p* = 0.048) and *Christensenellaceae* R7 group (< 0.001 % vs 0.846 ± 0.0648 %, *p* = 0.012).

The family *Bifidobacteriaceae* were significantly higher after including PPWS in the fermentation for 24 hrs when compared to cellulose (4.031 ± 0.270 % vs 2.708 ± 0.154 %, *p* = 0.001), whereas the families *Clostridiaceae* 1 and *Christensenellaceae* were significantly lower (1.400 ± 1.215 % vs 4.150 ± 0.738 %, *p* = 0.032; and < 0.001 % vs 1.812 ± 0.144 %, *p* = 0.017, respectively). The phylum Verrucomicrobia was significantly lower after fermentation with PPWS when compared to cellulose after 24 hrs (9.112 ± 0.441 % vs 11.735 ± 0.470 %, *p* = 0.002). LEfSe analysis determined that *Blastopirellula*, *Butyricimonas*, *Desulfovibrio*, *Kluyvera*, *Parabacteroides*, and an uncultured bacterium were discriminative bacterial genera of PPWS fermentation when compared to cellulose after 10 hrs (**Figure 5-13**). While *Ruminococcus* 1, *Bacteroides*, *Pseudobutyrivibrio*, *Bifidobacterium*, *Acidaminococcus*, *Mitsuokella*, *Phascolarctobacterium*, *Butyricimonas*, *Dorea*, *Enterobacter*, *Kluyvera*, *Bilophila*, *Citrobacter*, *Sutterella*, *Lachnospiraceae* UCG004, and *Parabacteroides* were discriminative bacterial genera of PPWS fermentation when compared to cellulose after 24 hrs (**Figure 5-13**).

By including PPE in the fermentation, there was a significantly higher relative abundance of the genus *Coprococcus* 2 after 10 hrs when compared to cellulose

(1.182 ± 0.161 % vs 0.387 ± 0.186 %, *p* = 0.005) (**Table 5-3**). After 24 hrs PPE fermentation, the relative abundance of the genera *Ruminococcaceae* UCG002 and *Barnesiella* was significantly lower when compared to cellulose (0.310 ± 0.536 % vs 1.146 ± 0.131 %, *p* = 0.032; and < 0.001 vs 0.950 ± 0.070 %, *p* = 0.021, respectively), whereas the relative abundance of the genera *Prevotella* 2 and *Bifidobacterium* was significantly higher when compared to cellulose (0.794 ± 0.198 % vs < 0.001 %, *p* = 0.035 and *p* = 0.001, respectively). Significantly higher relative abundances of the family *Bifidobacteriaceae* and the phylum Actinobacteria were also observed after 24 hrs (4.467 ± 0.252 % vs 2.708 ± 0.154 %, *p* = 0.001; and 13.79 ± 0.30 % vs 10.28 ± 0.17 %, *p* = < 0.001, respectively). The family *Enterococcaceae* were also significantly higher in vessels fermented with PPE when compared to cellulose after 24 hrs (5.29 ± 0.54 % vs 4.22 ± 0.17 %, *p* = 0.017). LEfSe analysis determined that *Coprococcus* 2, *Asteroleplasma*, *Enterococcus*, *Desulfovibrio*, *Pseudobutyrivibrio*, and two uncultured bacteria were discriminative bacterial genera of PPE fermentation when compared to cellulose after 10 hrs (). While *Bifidobacterium*, *Lachnospiraceae* UCG008, *Veillonella*, *Enterococcus*, and *Butyricimonas* were discriminative bacterial genera of PPE fermentation when compared to cellulose after 24 hrs (**Figure 5-14**).

The significant differences in bacterial composition following Synergy 1 fermentation when compared to cellulose are presented in **Table 5-4**. This included a significantly higher relative abundance of the *Bifidobacterium* genus after 24 hrs (2.362 ± 0.055 % vs 1.221 ± 0.082 %, *p* = < 0.001).

5.4.3.8 Short chain fatty acid and branched chain fatty acid analysis

Inclusion of PPWS in the fermentation resulted in significantly higher concentrations of propionic acid after 10 hrs when compared to cellulose (p = 0.014) (**Figure 5-15**). Inclusion of PPWS in the fermentation also resulted in significantly higher butyric acid concentrations after 24 hrs (p = 0.014), 36 hrs (p = 0.002), and 48 hrs (p = 0.001), and significantly higher valeric acid concentrations after 10, 24, 36, and 48 hrs when compared to cellulose (p = 0.018, p = 0.005, p = 0.002, and p = 0.049, respectively). Hexanoic acid was also significantly higher in PPWS treated vessels when compared to cellulose after 10, 24, and 36 hrs fermentation (p = 0.039, p = 0.012, and p = 0.012, respectively). Total SCFA were significantly higher in vessels when PPWS was included in the fermentation at 24 hrs and 36 hrs (p = 0.047 and p = 0.006, respectively).

Inclusion of PPWS in the fermentation resulted in significantly higher isobutyric acid concentrations after 24 and 36 hrs when compared to cellulose (p = 0.008 and p = 0.004, respectively) (**Figure 5-16**). Isovaleric acid concentrations were significantly higher when compared to cellulose after 5, 10, 24, and 36 hrs fermentation (p = 0.002, p = 0.017, p = 0.004, and p = 0.001, respectively). Total BCFA were significantly higher when including PPWS in the fermentation when compared to cellulose after 5, 10, 24, and 36 hrs fermentation (p = 0.004, p = 0.026, p = 0.005, and p = 0.002, respectively). Inclusion of neither Synergy 1 nor PPE in the fermentation resulted in a significant difference in the concentration of individual SCFA and total SCFA (**Figure 5-15**) or individual BCFAs and total BCFAs (**Figure 5-16**) at any time point during fermentation when compared to cellulose.

5.5 Discussion

In this study, we investigated the fermentability of the Irish red seaweed *P. palmata* (PPWS), and a polysaccharide-rich extract of *P. palmata* (PPE), and their impact on the microbial composition, diversity and metabolic activity of the human gut microbiota, using an *in vitro* human colonic fermentation model. In line with the properties of a prebiotic dietary fibre, the data obtained showed that individual and total SCFA concentrations were significantly higher in vessels that included PPWS in the fermentation when compared to the cellulose control. Evaluation of *Bifidobacterium* and *Lactobacillus*, traditionally associated with prebiotic activity, showed that both PPWS (culture and 16S rRNA amplicon sequencing) and PPE (qPCR and 16S rRNA amplicon sequencing) significantly stimulated *Bifidobacterium* populations but that neither PPWS nor PPE had any impact on the growth of *Lactobacillus* spp. (culture, qPCR, and 16S rRNA amplicon sequencing). Furthermore, 16S rRNA amplicon sequencing indicated that both seaweed substrates stimulated the growth of other bacterial genera when compared to cellulose, including *Bacteroides*, *Butyricimonas*, *Dorea*, *Parabacteroides*, *Phascolarctobacterium*, and *Pseudobutyrivibrio* (PPWS); and *Butyricimonas*, *Coprococcus*, *Lachnospiraceae* UCG008, and *Prevotella* (PPE), however neither substrate significantly altered the alpha or beta diversity

The fermentability of fibre present in PPWS and PPE is likely to be attributable to the presence of the digestion resistant complex hemicellulose polysaccharide, xylan, composed of 1,3- and 1,4- linked β-D-xylopyranose (Kobayashi et al., 2020), which has to capacity to be hydrolysed by carbohydrate active enzymes (CAZymes) such as β-(1→3)-xylosidases and α-L-arabinofuranosidases within a range of

bacterial species present in the human gut (Kobayashi et al., 2020). The fermentability and prebiotic potential of terrestrial-derived xylans, arabinoxylans, and their respective oligosaccharides, has previously been reported (Broekaert et al., 2011; Finegold et al., 2014; Lecerf et al., 2012; Lin et al., 2016; Walton et al., 2012; Yang et al., 2015), however the unique composition of *P. palmata* and the structural differences of its constituent xylan warranted this investigation. Further, the present study builds on the only previous *in vitro* fermentation study of *P. palmata*-derived xylan which was limited to the quantification of SCFA concentrations without any investigation of microbial compositional analysis (Lahaye et al., 1993).

The consistent bifidogenic effect of PPWS alongside the significantly higher concentrations of total SCFA, most notably propionate (10 hrs) and butyrate (24-48 hrs) in comparison to cellulose highlights its considerable potential as a prebiotic candidate. This suggests that *P. palmata* seaweed contains microbiota accessible components that are fermentable, which could potentially impact host physiology and benefit health.

A range of alterations to the microbiota were noted following PPWS treatment, many of which are likely to have contributed, in part, to the observed higher SCFA concentrations when compared to cellulose. This included acetic acid producers such as *Dorea* and *Phascolarctobacterium* (Duncan et al., 2007; Wu et al., 2017), propionic acid producers such as *Acidaminococcus*, *Phascolarctobacterium* and *Parabacteroides* (Jumas-Bilak et al., 2007; Wu et al., 2017, 2019), and butyric acid producers such as *Butyricimonas* and *Pseudobutyrivibrio* (Bailey et al., 2011; Neto and O'Toole, 2016). Theoretically, the latter could metabolise xylose into butyrate

via the pentose-phosphate pathway (Rivière et al., 2016). The *Pseudobutyrivibrio* and *Parabacteroides* genera have been associated with anti-inflammatory effects in murine models (Henson and Phalak, 2018; Kverka et al., 2011; Wu et al., 2019) whilst *Butyricimonas* has been shown to be more abundant in lean individuals (Garcia-Mantrana et al., 2018). Interestingly, enhanced *Parabacteroides* have been reported to be stimulated during an *in vitro* fermentation of brown seaweed substrates (Strain et al., 2019), but decreased in mice that consumed a sulphated polysaccharide from the red seaweed *Gelidium pacificum* (Cui et al., 2020). These substrate-specific differences on the genus *Parabacteroides* exemplifies the difficulty of comparing previous prebiotic screening studies using seaweeds.

The significant changes in *Bacteroides* associated with PPWS treatment may be important for the initial degradation of the polysaccharides present, considering the extensive glycobiome that *Bacteroides* spp. possess which makes them capable of degrading multiple complex dietary glycans (Ndeh and Gilbert, 2018). The presence of enzymes capable of degrading seaweed derived polysaccharide has been attributed to horizontal gene transfer of CAZymes from marine bacteria present on the seaweed vector (Hehemann et al., 2010; Ndeh and Gilbert, 2018; Schwalm and Groisman, 2017).

The significantly higher relative abundance of the marine bacterium *Blastopirellula* following PPWS treatment is likely to result from its presence on the seaweed and may contribute to the catabolism of the seaweed polysaccharides and contribute to cross-feeding of metabolites with the human gut commensals, including *Bacteroides* and *Bifidobacterium* (Lage and Bondoso, 2014; Turroni et al., 2015).

In contrast to the higher concentration of SCFA following PPWS fermentation, concentrations of isobutyric acid (24 and 36 h), isovaleric acid (5, 10, 24, and 36 h) and total BCFA (5, 10, 24, and 36 h) were significantly higher than cellulose. Given that *P. palmata* has a high protein content, previously shown to resist digestion *in vitro* (Galland-Irmouli et al., 1999), a potential explanation for the observed increase in BCFA during PPWS fermentation is that proteins were liberated from the seaweed food matrix and catabolised by the *ex vivo* bacterial community (alongside carbohydrates and other phytochemicals present in the seaweed food matrix). Given that *P. palmata* could provide a non-animal source of dietary protein, further investigation to understand the concentrations of protein fermentation metabolites such as hydrogen sulphide, ammonia, and *p*-Cresol are needed, owing to their potential genotoxic effects (Diether and Willing, 2019).

Proteolytic fermentation may also explain why PPWS was associated with the genus *Bilophila* (LEfSe analysis). *Bilophila* spp. produce hydrogen sulphide via taurine deconjugation (Xing et al., 2019), and red seaweeds such as *P. palmata* are reported to contain a high amount of the amino acid taurine (Kawasaki et al., 2017). Given the pathobiont nature of certain *Bilophila* species, the impact of *P. palmata* seaweed consumption on the production of hydrogen sulphide, a mediator of gut homeostasis in both physiology and pathophysiology (Wallace et al., 2017), warrants further exploration. Similarly, the impact of PPWS on the potentially pathogenic genera, *Citrobacter* and *Kluyvera* should be considered.

The inclusion of PPE in the fermentation was shown to exert bifidogenic effects as noted by the qPCR and 16S rRNA amplicon sequencing data in the absence of higher SCFA concentrations when compared to cellulose. The lack of significantly

higher bifidobacteria by the culture dependent quantification following PPE treatment raises a question mark over the comparison of genomic approaches and viable counts (Gloor et al., 2017; Hawinkel et al., 2019). The bifidogenic effect has been proposed to be an important factor in the health benefits associated with prebiotics because bifidobacteria are suggested to exert health promoting effects via immunomodulatory enhancement of regulatory T-cell function (Ruohtula et al., 2019; Verma et al., 2018), the synthesis of vitamins B & K (Rossi et al., 2011; Yoshii et al., 2019), and also produce bacteriocins (Rivière et al., 2016). A recent study by Kobayashi *et al.* (Kobayashi et al., 2020) has reported that *Bifidobacterium adolescents* expresses β-(1→3)-xylosidases and α-L-arabinofuranosidases that degrade *P. palmata* derived xylan. This may help to explain, in part, the bifidogenic effect observed in this study.

PPE fermentation did not stimulate the growth of *Lactobacillus* populations when compared to cellulose. These data are in agreement with one previous human intervention study which demonstrated significantly increased faecal bifidobacteria but not lactobacilli following xylo-oligosaccharide (XOS) treatment (Finegold et al., 2014), but conflicts partly with another study which reported significant increases in both lactobacilli and bifidobacteria (Lin et al., 2016).

The genus *Prevotella* 2 was also significantly higher in PPE treated vessels when compared to cellulose. *Prevotella*, of the Bacteroidetes phylum, are associated with high dietary fibre diets (O'Grady et al., 2019). *Prevotella* possess xylan utilisation system (XUS) gene clusters and glycoside hydrolase enzymes, and therefore *Prevotella* spp. may have the potential to be xylanolytic via the xylose isomerase pathway to produce SCFA such as acetate and propionate, and also facilitates

cross-feeding for other bacteria (Dodd et al., 2011; Franke and Deppenmeier, 2018; Poeker et al., 2018).

Although the PPE treatment had no significant impact on SCFA concentration, several bacterial genera were stimulated by PPE treatment when compared to cellulose. This included *Lachnospiraceae* UCG008, *Veillonella*, *Enterococcus* and *Butyricimonas*. Of note, *Butyricimonas* produce butyric acid (Garcia-Mantrana et al., 2018), while *Veillonella* produce propionic acid (Henson and Phalak, 2018). *Lachnospiraceae* are common gut commensals capable of degrading plant biopolymers (Biddle et al., 2013; Esquivel-Elizondo et al., 2017; Tidjani Alou et al., 2016) and are increased following high-fibre diets (Bishehsari et al., 2018).

The higher concentrations of SCFA following PPWS treatment in comparison with the lack of any effect in the PPE treatment makes the PPWS a more suitable prebiotic source as a range of health benefits associated with fermentable polysaccharides have been attributed to SCFA production. For instance, SCFA are involved in both local and systemic functions *in vivo*, including pathogen inhibition (owing to decreased luminal pH), hepatic gluconeogenesis (propionic acid), and the hormonal control of satiety (promotion of CCK, PYY and GLP-1 release) (Canani et al., 2011; Frost et al., 2014; González Hernández et al., 2019; Halford and Harrold, 2012). Further, butyric acid is the primary energy source for colonocytes energy and is understood to exert anti-cancer effects through its histone deacetylase inhibitory activities (Leonel and Alvarez-Leite, 2012; Plöger et al., 2012; Velazquez et al., 1997), as well as anti-inflammatory effects via $Foxp3^{+}$ Treg cell proliferation and NF-κB transcription factor inhibition (Leonel and Alvarez-Leite, 2012; Plöger et al., 2012).

Cellulose was selected as the negative control in this study because it is poorly fermented by the human gut microbiota and is not a prebiotic (Gibson et al., 2017). Nevertheless, PPWS, PPE, and Synergy 1 treated vessels had a significantly lower relative abundance of the genus *Ruminococcaceae* UCG002 when compared to cellulose after 24 hrs. *Ruminococcus* spp. are cellulolytic (Chassard et al., 2010; Flint et al., 2012).

The whole seaweed had a more profound effect on SCFA production than PPE, which suggests that the synergy of multiple components present in the seaweed matrix is required for faecal bacterial communities to thrive within a closed, batch-culture fermentation system. This was also observed in a recent *in vitro* fermentation study whereby whole oats increased bifidobacteria, acetic acid, and propionic acid to a greater extent than extracted oat beta glucans and oat polyphenols (Kristek et al., 2019). Nevertheless, the higher iodine content of PPWS may limit the amount of seaweed which could be safely consumed in order to test prebiotic efficacy *in vivo*, where a 5g portion of PPWS would exceed the 600 µg/day and 1100 µg/day upper limits set by the European Food Safety Authority and the World Health Organisation, respectively (EFSA, 2014; Joint FAO/WHO Expert Committee on Food Additives, 1989). Indeed, one previous study found that supplementation of 5g/day *P. palmata* for 28 days improved iodine status in adults but it significantly increased serum thyroid stimulating hormone albeit within normal clinical ranges (Allsopp et al., 2016). Therefore, optimised methods which extract and purify the fibre content of *P. palmata* warrants further investigation, given that the observed stimulation of *Bifidobacterium* and *Prevotella* by PPE when compared to cellulose.

5.6 Conclusion

The data obtained from this *in vitro* study provide evidence that the edible red seaweed *P. palmata*, contains microbiota accessible components for fermentation by human faecal bacterial populations. The results of this study indicate that the *P. palmata* whole seaweed can favourably impact microbiota composition and metabolic output, whilst the changes induced by the *P. palmata* polysaccharide extract were limited to bacterial composition, albeit a bifidogenic effect was observed. PPWS would appear to be a food that promotes a health-associated gut microbiota; however, its high iodine concentration needs to be considered when preparing this for human consumption. Further research is needed to clarify whether crude polysaccharide extracts, purified xylan or xylo-oligosaccharides, or other phytochemicals such as polyphenols or polyunsaturated fatty acids obtained from *P. palmata*, can positively modulate gut microbiota composition and function to confer health benefits conducive to a prebiotic *in vivo*.

5.7 References

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5.8 Figures

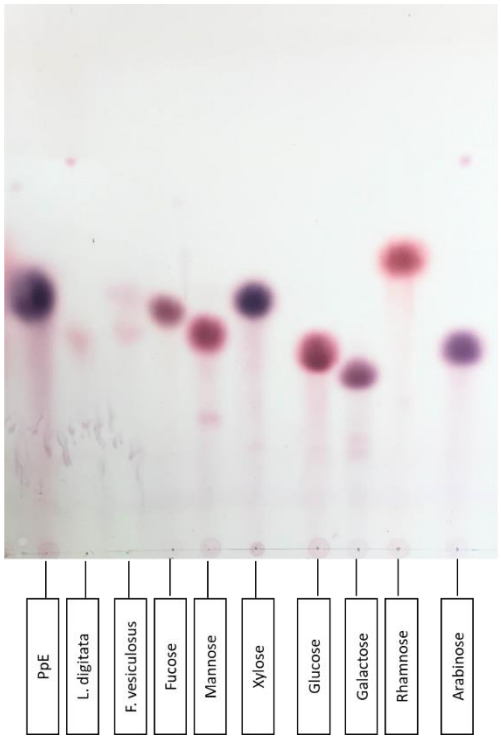

Figure 5-1. Silica-gel thin-layer chromatography (TLC) of PPE alongside monosaccharide standards of fucose, mannose, xylose, glucose, galactose, rhamnose, and arabinose.

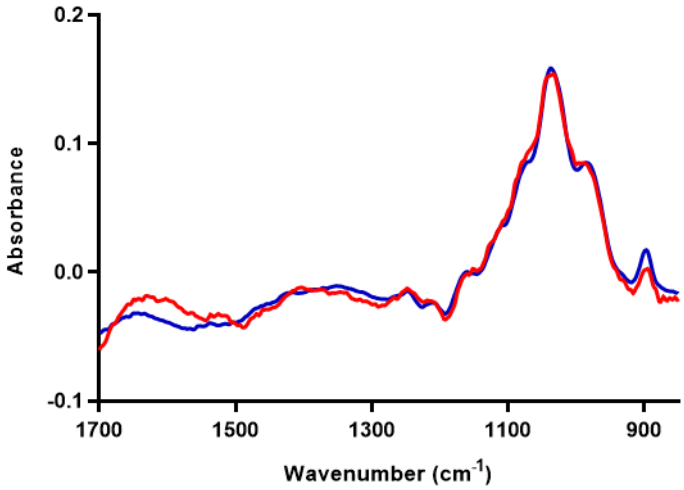

Figure 5-2 Structural information of the *P. palmata* polysaccharide extract was determined using Fourier transform infrared spectroscopy (FTIR). Blue denotes the extract before *in vitro* digestion and red denotes the extract after *in vitro* digestion.

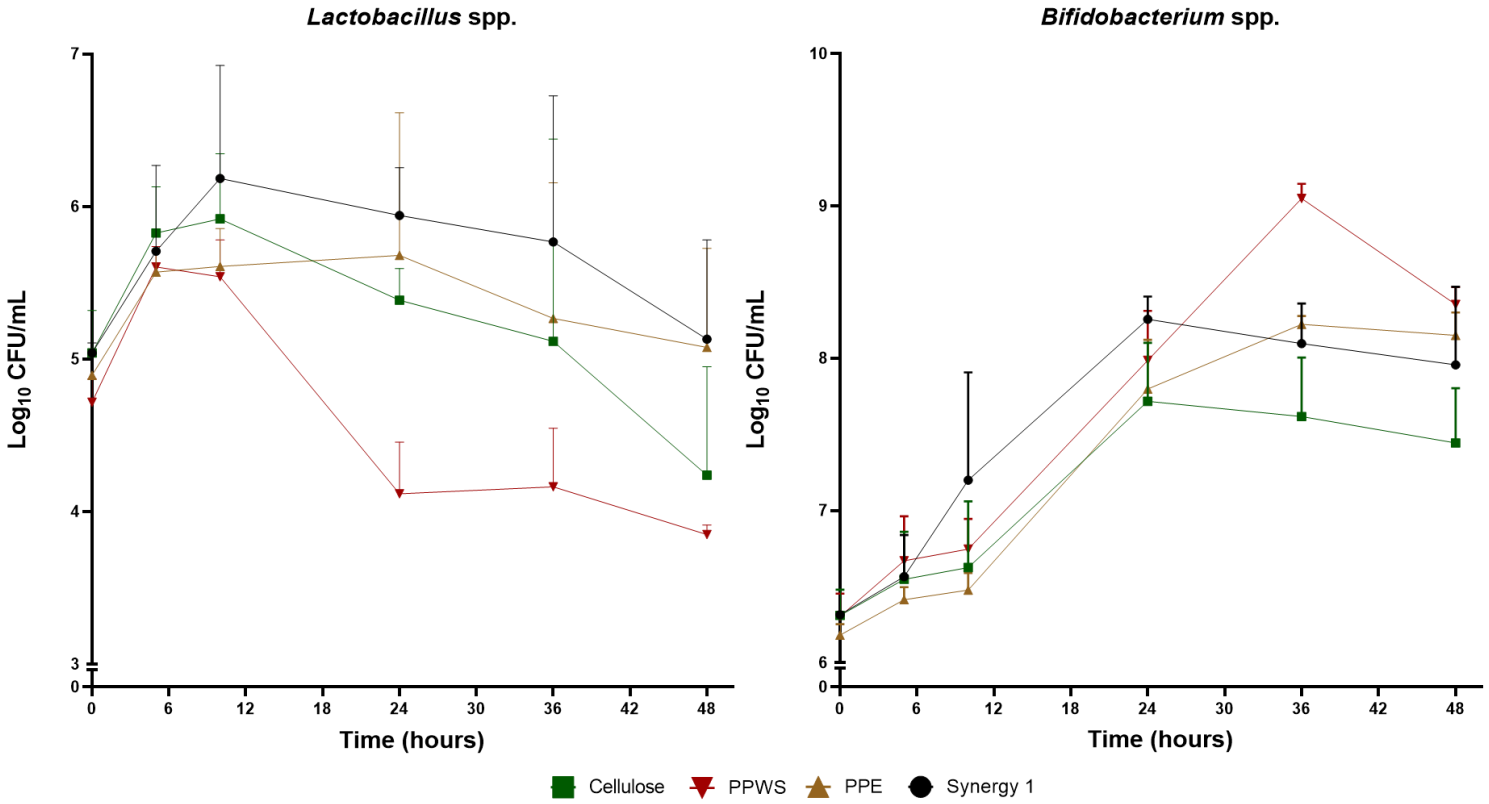

Figure 5-3. Bacterial enumeration (Log_{10} CFU/mL) of *Lactobacillus* spp. (LBS agar) and *Bifidobacterium* spp. (MRS agar) present in fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* whole seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard deviation (n = 3). Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q-value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

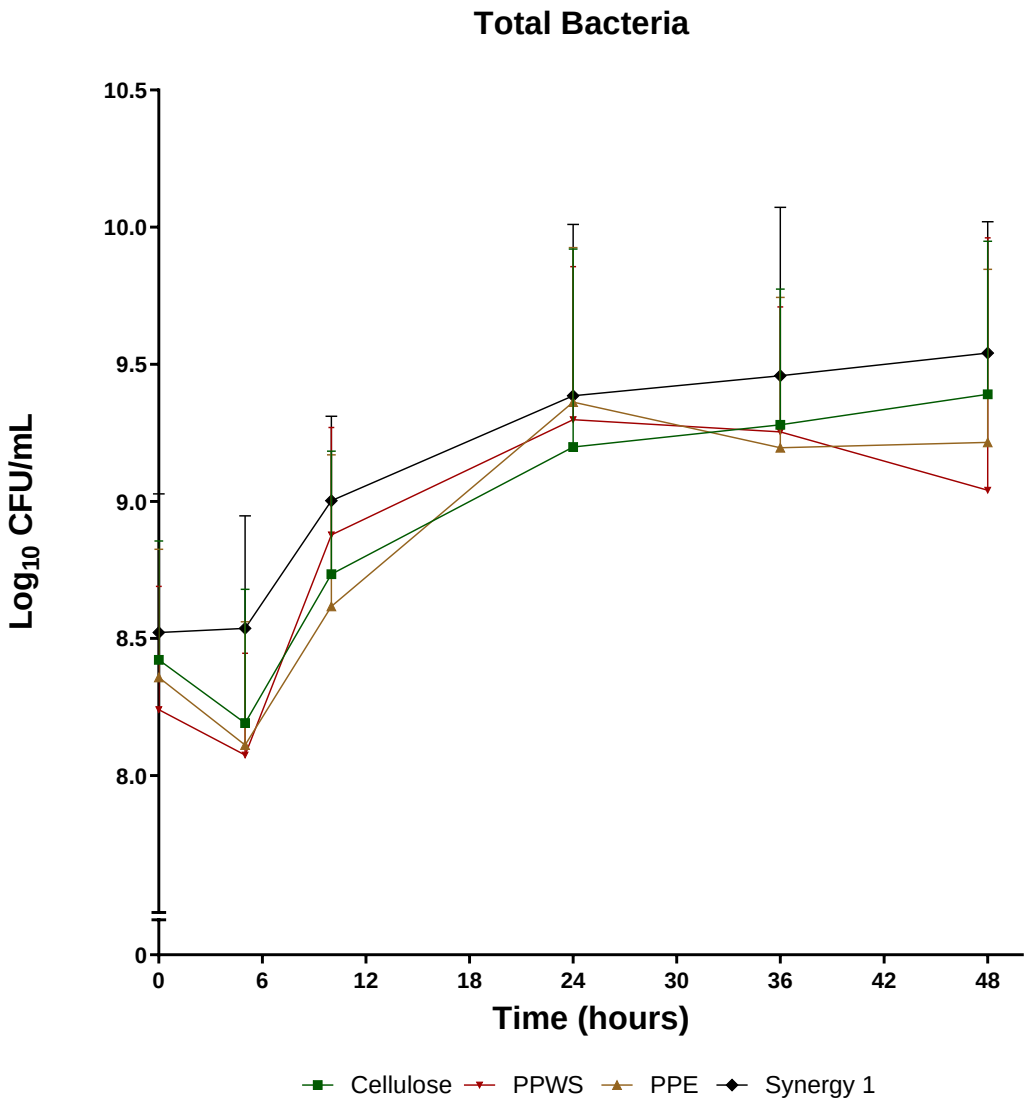

Figure 5-4. qPCR was used to quantify total bacteria from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* whole seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q-value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

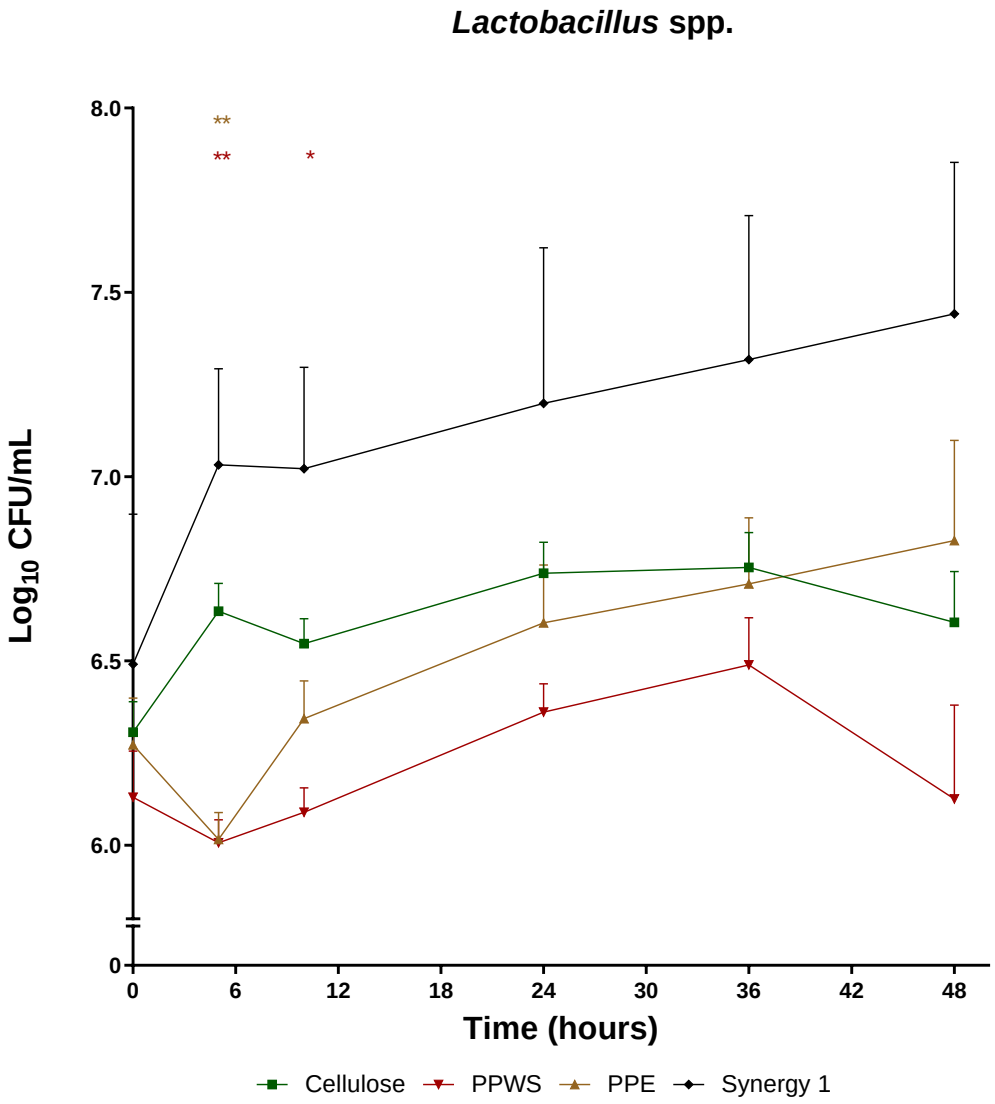

Figure 5-5. qPCR was used to quantify *Lactobacillus* spp. from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* whole seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q-value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

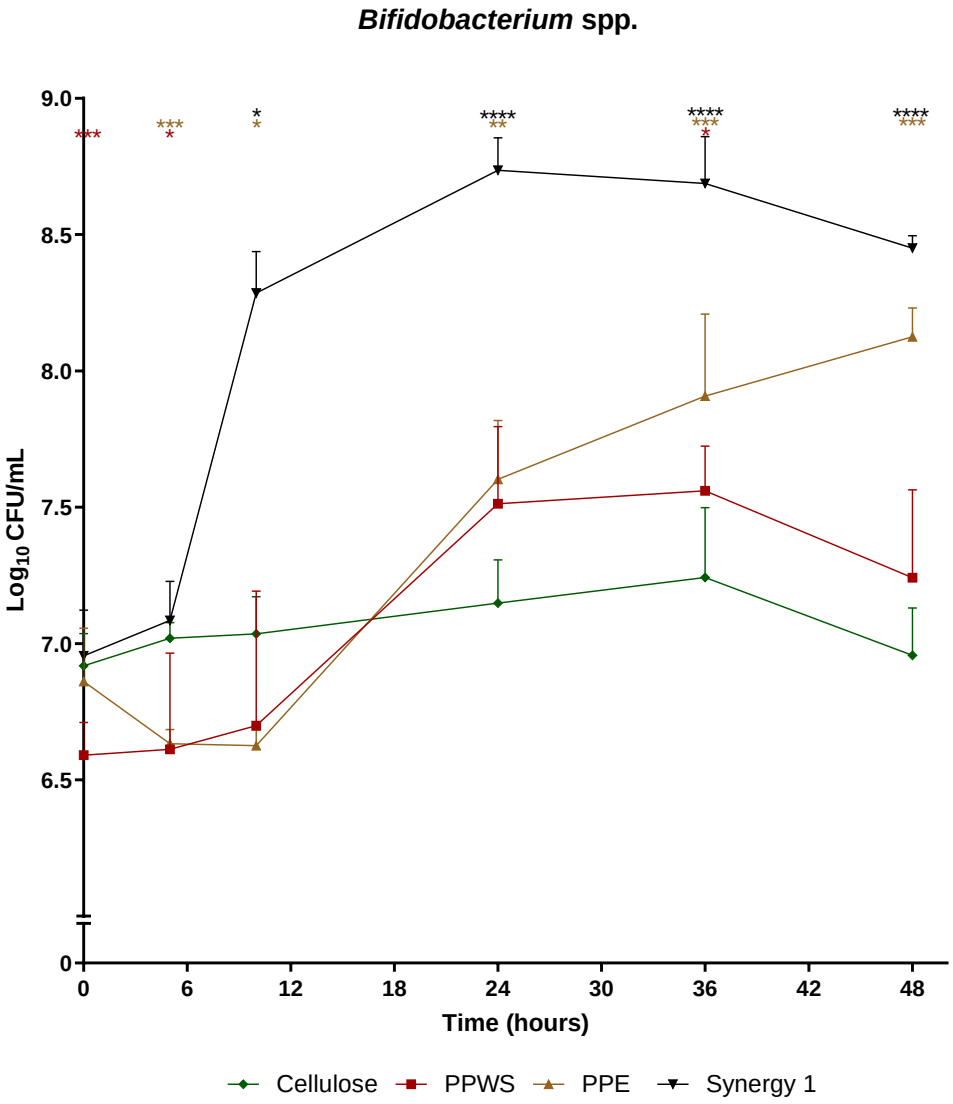

Figure 5-6. qPCR was used to quantify *Bifidobacterium* spp. from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* whole seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 substrates. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q-value). * q ≤ 0.05, ** q ≤ 0.01, *** q ≤ 0.005, **** q ≤0.0001.

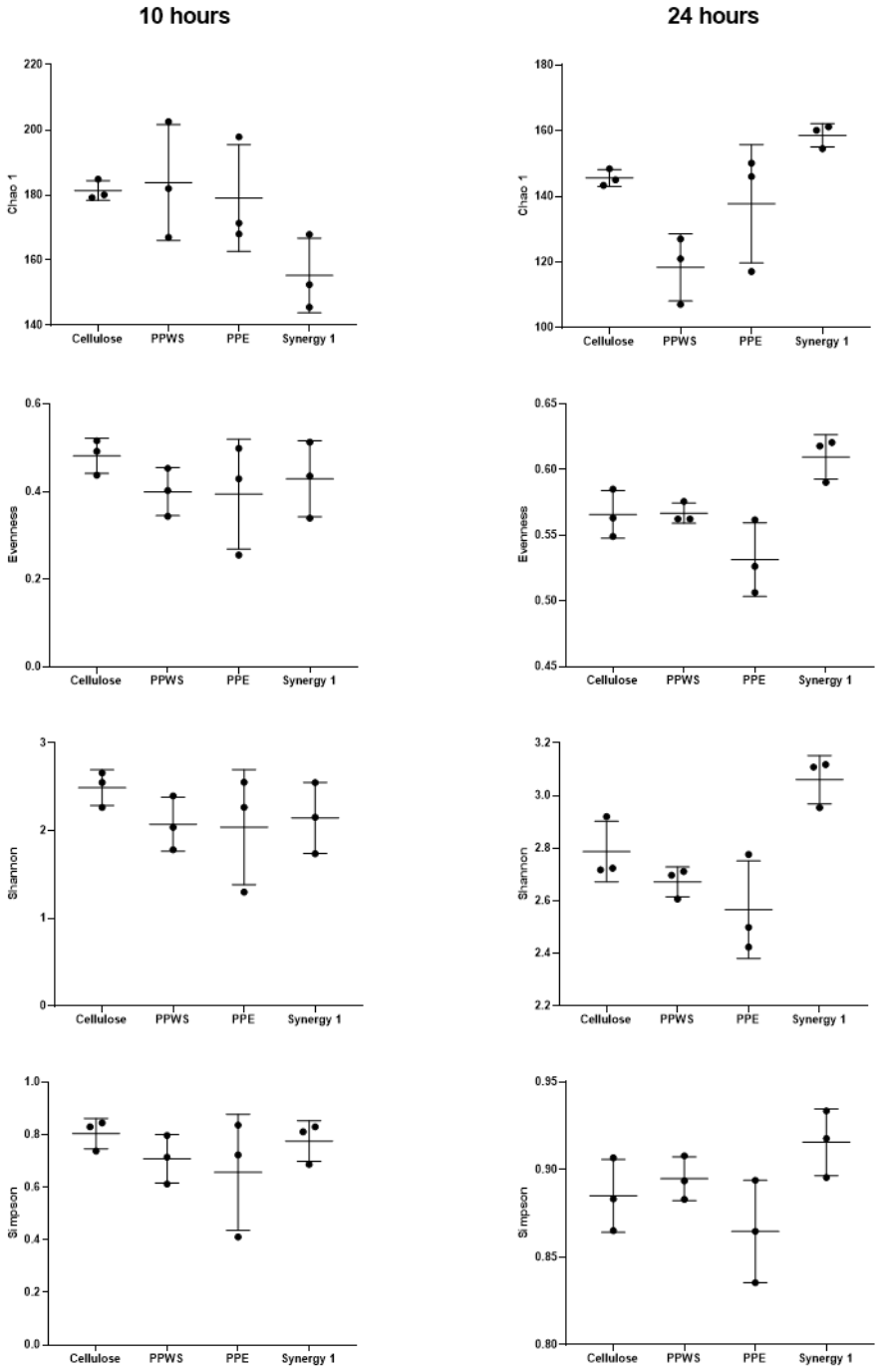

Figure 5-7. Chao1, Evenness, Shannon and Simpson alpha diversity indices of fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* whole seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1, after 10 hrs and 24 hrs. Plots represent individual values and the mean ± one standard deviation of the mean (n = 3). Statistical significance was accepted as $p \leq 0.05$ following a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons.

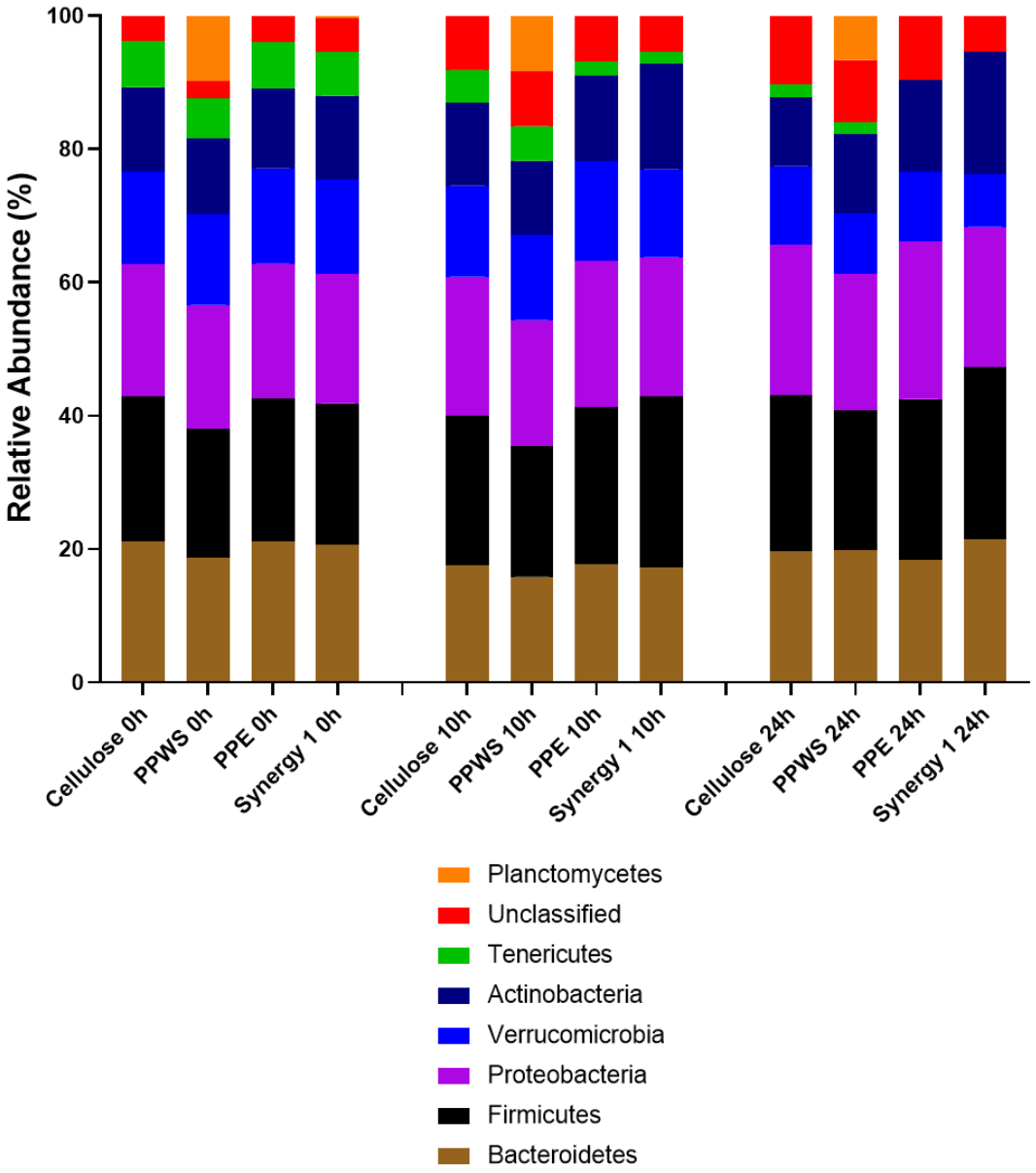

Figure 5-8. Mean relative abundances of bacterial phyla in fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 after 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

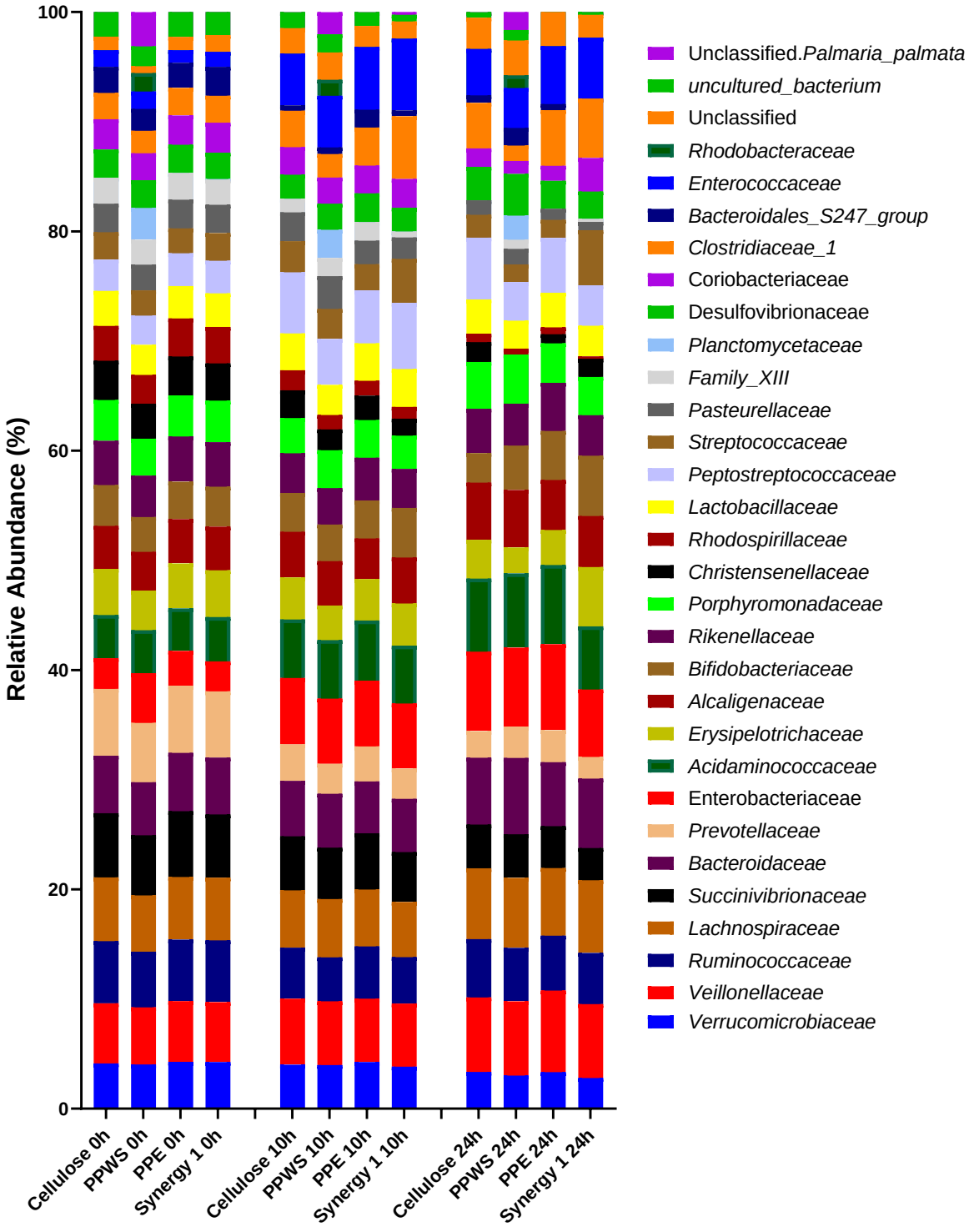

Figure 5-9. Mean relative abundances of bacterial families in fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 after 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

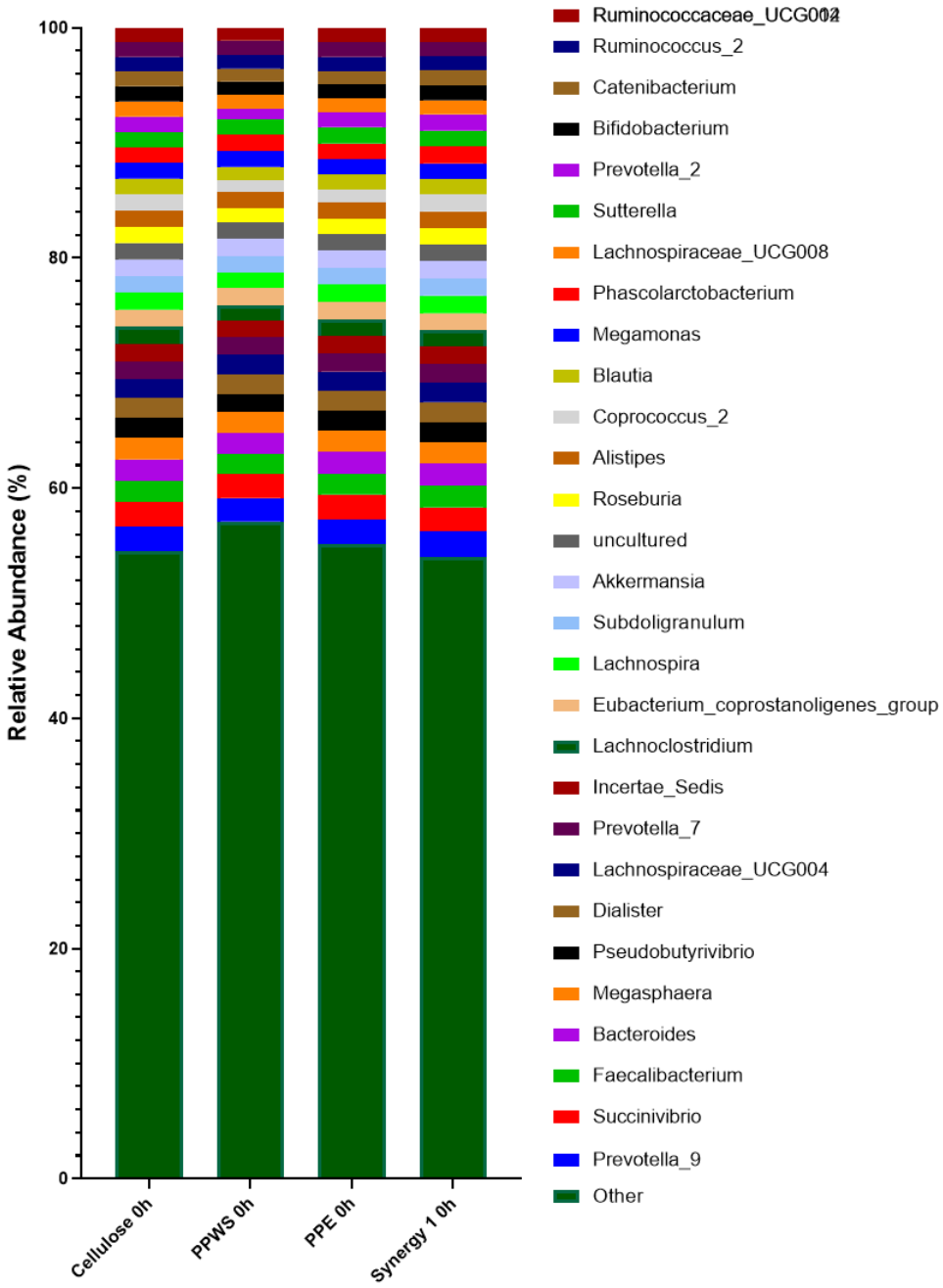

Figure 5-10. Mean relative abundances of bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 after 0 hrs fermentation (n = 3). The top 30 most abundant genera are represented as individual stacks with all remaining genera assigned a single stack labelled “other”. Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

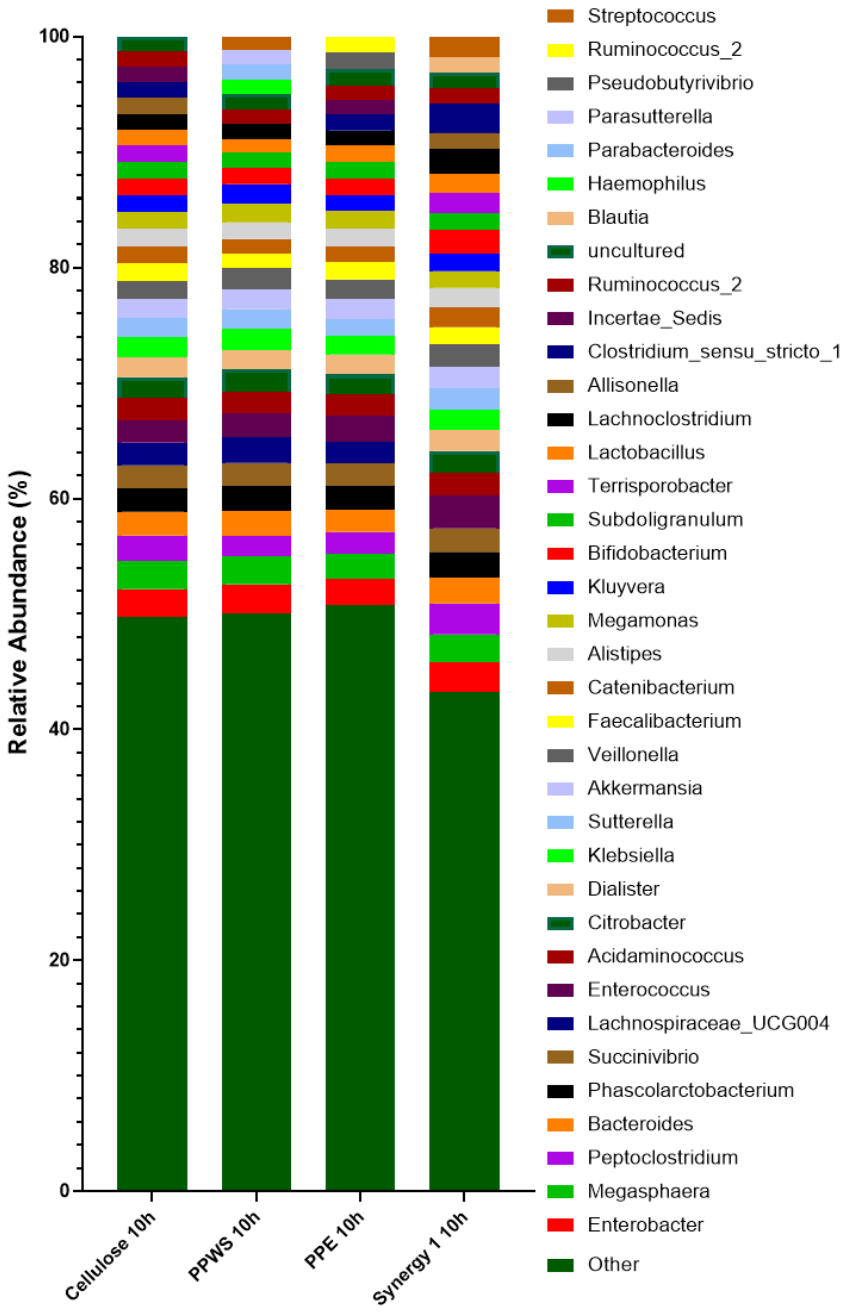

Figure 5-11. Mean relative abundances of bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 after 10 hrs fermentation (n = 3). The top 30 most abundant genera are represented as individual stacks with all remaining genera assigned a single stack labelled “other”. Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

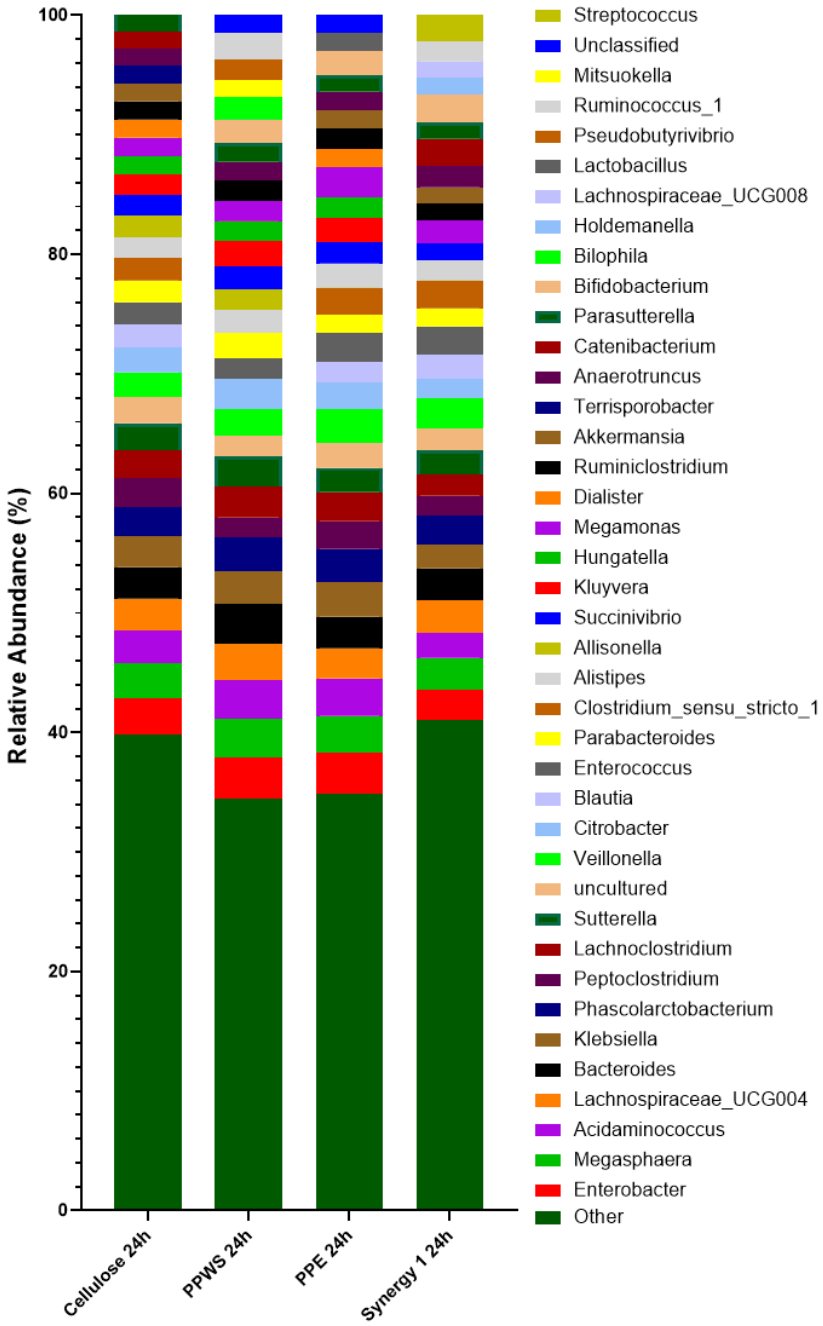

Figure 5-12. Mean relative abundances of bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1 after 24 hrs fermentation (n = 3). The top 30 most abundant genera are represented as individual stacks with all remaining genera assigned a single stack labelled “other”. Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

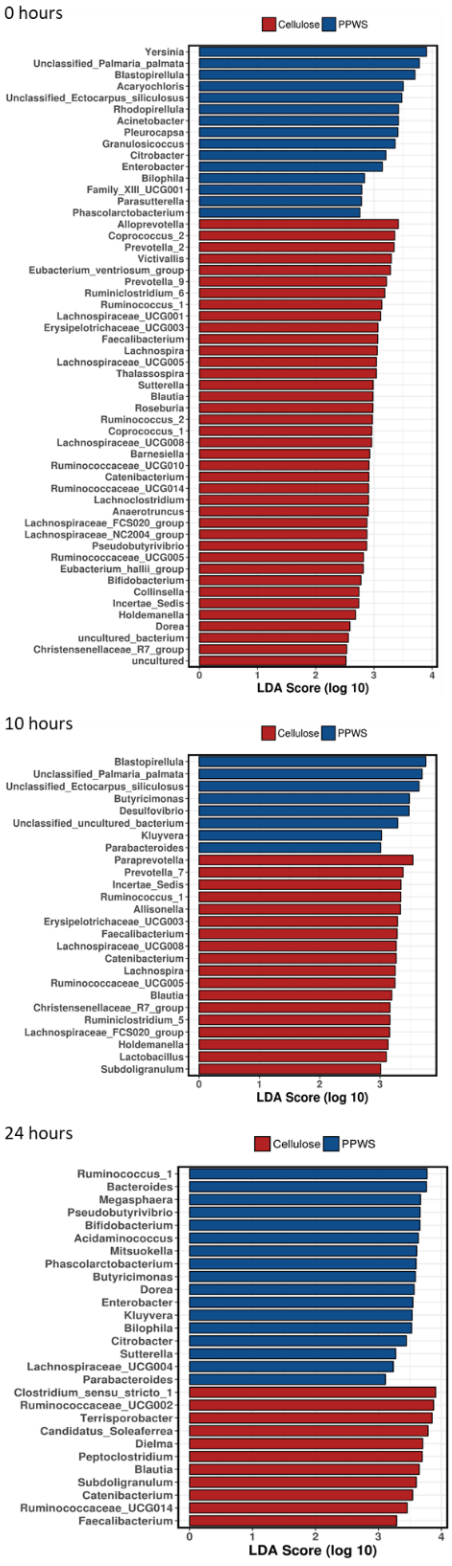

Figure 5-13. LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) *P. palmata* whole seaweed (PPWS) when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

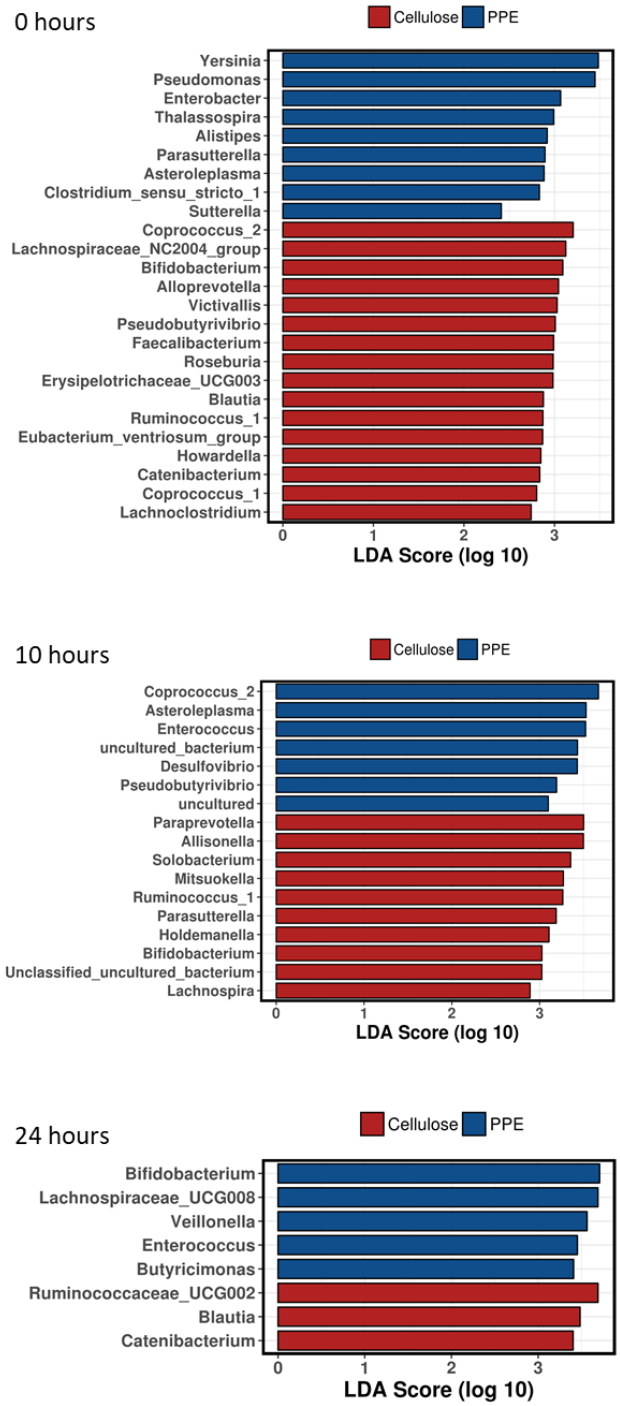

Figure 5-14. LDA scores following LEfSe analysis to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) *P. palmata* polysaccharide extract (PPE) when compared to 1% (w/v) cellulose at 0, 10, and 24 hrs fermentation (n = 3). Bacterial composition was determined using 16S rRNA amplicon sequencing on the MiSeq platform.

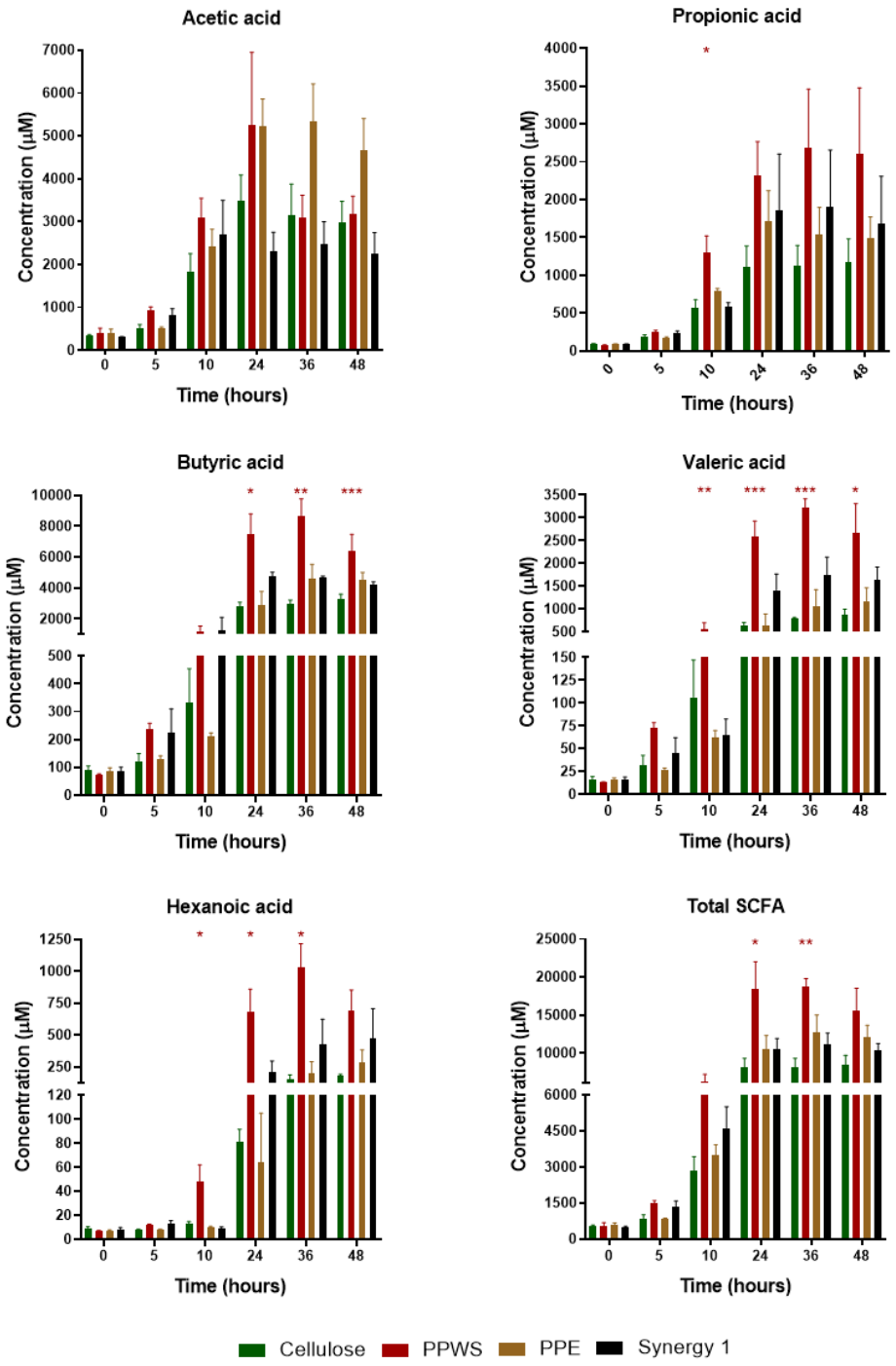

Figure 5-15. SCFA concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* whole seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean (n = 3). Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

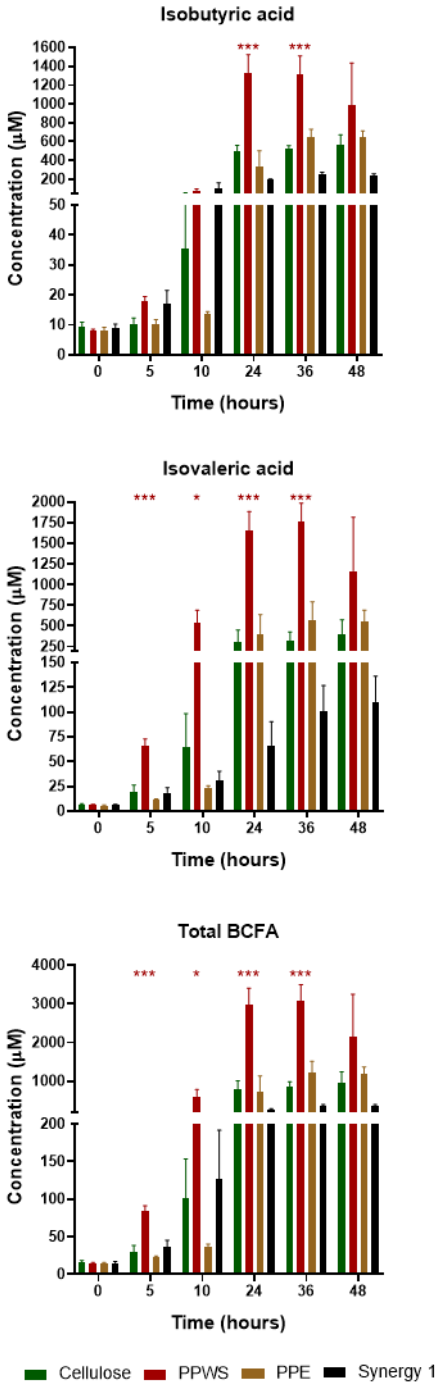

Figure 5-16. BCFA concentrations (µM) of fermentation vessels treated with either 1% (w/v) cellulose, *P. palmata* whole seaweed (PPWS), *P. palmata* polysaccharide extract (PPE), or Synergy 1. Samples were taken at 0, 5, 10, 24, 36, and 48 hrs fermentation. Data plots represent the mean + standard error of the mean. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ when compared to cellulose at each time point.

5.9 Tables

Table 5-1. Mineral and trace element contents of the *Palmaria palmata* whole seaweed (PPWS) (n = 1) and *Palmaria palmata* polysaccharide rich extract (PPE) (n = 1) batch culture fermentation substrates. ppm – parts per million; RNI - Reference Nutrient Intake.

Palmaria palmata	**Whole Seaweed**					**Polysaccharide Rich Extract**				
Mineral / Trace Element	**Concentration (ppm)**	**Equivalent in mg/g**	**Amount (mg) in 5g**	**Amount (mg) in 10g**	**% RNI (mg/day)**	**Concentration (ppm)**	**Equivalent in mg/g**	**Amount (mg) in 5g**	**Amount (mg) in 10g**	**% RNI (mg/day)**
Aluminium	151	0.151	0.755	1.51	Not applicable	35.4	0.0354	0.177	0.354	Not determined
Chloride	73435	73.44	367.18	734.4	29.374	6830	6.83	34.15	68.3	2.73
Copper	5.25	0.00525	0.0263	0.0525	4.375	10.1	0.0101	0.0505	0.101	8.42
Mercury	0.029	0.000029	0.000145	0.00029	Not applicable	0.004	0.000004	0.00002	0.00004	Not applicable
Lead	3.961	0.00396	0.0198	0.0396	Not applicable	1.147	0.00115	0.00574	0.0115	Not applicable
Arsenic	10.36	0.0104	0.0518	0.104	Not applicable	3.472	0.00347	0.0174	0.0347	Not applicable
Cadmium	0.344	0.000344	0.00172	0.00344	Not applicable	0.184	0.000184	0.00092	0.00184	Not applicable
Iodine	519.2	0.519	2.60	5.19	3461.33	150	0.15	0.75	1.5	1000
Sodium	13200	13.2	66	132	8.25	3132	3.132	15.66	31.32	1.96
Zinc	48.2	0.0482	0.241	0.482	5.074	13.3	0.0133	0.0665	0.133	1.4

Table 5-2. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *P. palmata* whole seaweed (PPWS) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		PPWS		Effect	*P*-Value
		Mean (%)	SD	Mean (%)	SD		
	Phylum						
24 h	Verrucomicrobia	11.735	0.47	9.112	0.441	↓	0.002
	Family						
10 h	*Erysipelotrichaceae*	3.845	0.222	3.152	0.269	↓	0.038
24 h	*Clostridiaceae* 1	4.15	0.738	1.4	1.215	↓	0.032
	Christensenellaceae	1.812	0.144	< 0.001	< 0.001	↓	0.017
	Bifidobacteriaceae	2.708	0.154	4.031	0.27	↑	0.001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 5-2 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *P. palmata* whole seaweed (PPWS) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		PPWS		Effect	*P*-Value
		Mean (%)	SD	Mean (%)	SD		
Genus							
10 h	*Tyzzerella* 3	0.435	0.186	< 0.001	< 0.001	↓	0.006
	Lachnospiraceae FCS020 group	0.862	0.091	0.608	0.108	↓	0.03
	Faecalibacterium	1.519	0.06	1.211	0.127	↓	0.021
	Erysipelotrichacea UCG003	0.996	0.059	0.655	0.099	↓	0.003
	Christensenellaceae R7 group	1.03	0.082	0.81	0.081	↓	0.028
	Alloprevotella	< 0.001	< 0.001	0.458	0.066	↑	0.005
24 h	*Ruminococcaceae* UCG002	1.146	0.131	< 0.001	< 0.001	↓	0.006
	Pseudobutyrivibrio	1.046	0.14	1.688	0.235	↑	0.021
	Clostridium sensu stricto 1	1.823	0.308	0.687	0.61	↓	0.048
	Christensenellaceae R7 group	0.846	0.0648	< 0.001	< 0.001	↓	0.012
	Butyricimonas	0.881	0.0125	1.448	0.212	↑	0.021
	Bifidobacterium	1.221	0.0819	1.955	0.184	↑	0.002
	Bacteroides	2.629	0.061	3.365	0.286	↑	0.007

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 5-3. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) *P. palmata* polysaccharide extract (PPE) when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		PPE		Effect	*P*-Value
		Mean (%)	SD	Mean (%)	SD		
	Phylum						
24 h	Actinobacteria	10.280	0.172	13.791	0.302	↑	0.001
	Family						
24 h	*Enterococcaceae*	4.221	0.166	5.294	0.539	↑	0.017
	Bifidobacteriaceae	2.708	0.154	4.467	0.252	↑	0.000153
	Genus						
10 h	*Coprococcus* 2	0.387	0.186	1.182	0.161	↑	0.005
24 h	*Ruminococcaceae*	1.146	0.131	0.310	0.536	↓	0.032
	Prevotella 2	< 0.001	< 0.001	0.794	0.198	↑	0.035
	Barnesiella	0.950	0.070	< 0.001	< 0.001	↓	0.021
	Bifidobacterium	1.221	0.082	2.056	0.221	↑	0.001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 5-4. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) Synergy 1 when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

			Cellulose		Synergy 1		Effect	*P*-Value
			Mean (%)	SD	Mean (%)	SD		
Phylum								
10 h	Firmicutes		22.409	0.942	25.557	1.563	↑	0.036
24 h	Verrucomicrobia		11.735	0.470	7.901	0.895	↓	< 0.0001
	Unclassified		10.352	0.203	5.349	1.781	↓	0.003
	Firmicutes		23.346	1.110	25.823	0.820	↑	0.045
	Actinobacteria		10.280	0.172	18.256	1.066	↑	< 0.0001
Family								
10 h	*Enterococcaceae*		4.710	0.319	6.533	0.542	↑	0.002
24 h	*Verrucomicrobiaceae*		3.355	0.057	2.804	0.288	↓	0.035
	Succinivibrionaceae		3.986	0.223	2.936	0.177	↓	0.015
	Streptococcaceae		2.075	0.317	5.019	0.231	↑	0.007
	Erysipelotrichaceae		3.529	0.259	5.408	0.623	↑	0.009
	Enterococcaceae		4.221	0.166	5.544	0.280	↑	0.005
	Bifidobacteriaceae		2.708	0.154	5.510	0.330	↑	< 0.0001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 5-4 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) Synergy 1 when compared to 1% (w/v) cellulose, after 10- and 24-hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p \leq 0.05$.

		Cellulose		Synergy 1		Effect	*P*-Value
		Mean (%)	SD	Mean (%)	SD		
	Genus						
10 h	*Tyzzerella* 3	0.435	0.186	0.000	0.000	↓	0.006
	Ruminiclostridium 5	0.800	0.109	0.135	0.233	↓	0.003
	Enterococcus	1.917	0.051	2.873	0.140	↑	< 0.0001
	Christensenellaceae R7 group	1.030	0.082	0.820	0.018	↓	0.035
24 h	*Streptococcus*	0.957	0.112	2.186	0.107	↑	0.020
	Ruminococcus 2	1.007	0.153	0.578	0.254	↓	0.041
	Lachnoclostridium	2.364	0.113	1.800	0.112	↓	0.001
	Bifidobacterium	1.221	0.082	2.362	0.055	↑	< 0.0001
	Anaerostipes	0.906	0.081	1.722	0.291	↑	0.032
	Acidaminococcus	2.784	0.117	2.031	0.148	↓	0.040

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Chapter 6

Experimental Chapter

6 Effects of seaweed polysaccharides on the composition and metabolic activity of the human gut microbiota using an *in vitro* model of the distal colon

6.1 Abstract

Seaweed has been proposed as a source of potential prebiotic fibres, however, limited studies have evaluated the prebiotic potential of individual seaweed derived polysaccharides. This study used an *in vitro* model of distal colonic fermentation to investigate the prebiotic potential of commercially available alginate, fucoidan, laminarin, ulvan, and xylan complex polysaccharides, isolated from seaweeds, by measuring changes in the composition (16S rRNA amplicon sequencing and qPCR analysis) and metabolic activity (short chain fatty acids) of the human gut microbiota. Prebiotic potential was compared to cellulose as a negative control. qPCR analysis showed that treatment with laminarin and xylan substrates resulted in significantly higher numbers of total bacteria and *Bifidobacterium* spp., respectively. 16S rRNA amplicon sequencing data showed significantly higher relative abundance of the *Bifidobacterium* genus for xylan, *Bacteroides* for alginate, and *Lachnospiraceae* for laminarin, when compared to cellulose. No significant changes in bacterial genera were noted for fucoidan and ulvan substrates. Xylan fermentation resulted in significantly higher concentrations of acetic, propionic, valeric and total short chain fatty acids, whilst laminarin and ulvan fermentation resulted in significantly higher concentrations of propionic acid and acetic acid, respectively. One of the two alginate substrates resulted in significantly higher concentrations of butyric acid. Fucoidan showed limited fermentability and showed no significant changes in bacterial composition. In

summary, most seaweed polysaccharides are fermentable, with xylan and laminarin showing the most promise as prebiotic polysaccharides derived from seaweed.

6.2 Introduction

Seaweed consumption has been associated with a reduce risk of chronic diseases including cancer, hyperlipidaemia, and coronary heart disease (Brown et al., 2014), which has led to increasing interest in seaweed as a source of functional food ingredients. Seaweeds are a source of bioactive compounds, including polyphenols such as phlorotannins, polyunsaturated fatty acids, vitamins and minerals (MacArtain et al., 2007). There is also considerable interest in the potential impact of the fibre components of red, brown and green seaweeds on health. Dietary fibre can make up to 75% of seaweed dry weight and contains a diverse range of complex polysaccharides which are generally not present in terrestrial plants. The fibres include alginate, fucoidan, and laminarin in brown seaweeds; ulvan in green seaweeds; and xylan in red seaweeds (O'Sullivan et al., 2010).

Interest in the biological activity of seaweed-derived complex polysaccharides is predominantly focused on its potential prebiotic effects through the selective stimulation of microbes deemed to be beneficial to health such *Bifidobacterium*, *Lactobacillus*, and other species that contribute to the production of health-associated short chain fatty acids (SCFA) (Brown et al., 2014). Accumulating evidence from *in vitro* fermentation studies indicates that whole seaweeds and polysaccharide extracts may favourably modulate gut microbial composition and increase the production of SCFA (Bajury et al., 2017; Chen et al., 2018; Di et al.,

2018; Fu et al., 2018; Kong et al., 2016; Rodrigues et al., 2016). The evidence, however, from *in vivo* animal models is inconclusive of a prebiotic effect (Bhattacharya et al., 2015; Charoensiddhi et al., 2017; Huebbe et al., 2017; Ren et al., 2017) and the limited number of studies which have assessed the fermentation of purified seaweed complex carbohydrates such as alginate, laminarin, fucoidan, and agar, present mixed results (An et al., 2013; Bai et al., 2017; Belanche et al., 2016; Devillé et al., 2007; Nakata et al., 2016; Ramnani et al., 2012; Seong et al., 2019; Shang et al., 2016). Given the structural diversity of polysaccharides present in seaweed there is a need to characterise the impact of each of these substrates on the composition and metabolism of the microbiota to determine their suitability as prebiotic candidates (O'Sullivan et al., 2010). Therefore, the aim of this study was to investigate the effects of alginate, fucoidan, laminarin, xylan and ulvan seaweed polysaccharides on the composition and metabolic activity of the *ex vivo* human gut microbiota, using an *in vitro* fermentation model of the distal colon, following a simulated *in vitro* digestion.

6.3 Materials and methods

6.3.1 Chemicals and reagents

Chemicals were purchased from Sigma Aldrich (St. Louis, USA) unless otherwise stated. Reagents used during High Performance Size Exclusion Chromatography (HPSEC) and Gas Chromatography Mass Spectrometry (GC-MS) were HPLC grade. Reagents used for DNA extraction, quantitative PCR (qPCR), and 16S rRNA next generation sequencing were molecular biology grade.

6.3.3 Seaweed polysaccharides

Fucoidan from *Fucus vesiculosus* (F5631) and Laminarin from *Laminaria digitata* (L9634) were purchased from Sigma Aldrich (USA). Alginate from *Fucus vesiculosus* (ALG101), Alginate from *Laminaria japonica* (ALG100), Fucoidan from *Ascophyllum nodosum* (FUC400), Ulvan from *Ulva* spp. (ULV100), and Xylan from *Palmaria palmata* (XYL100) were purchased from Elicityl (France). These substrates were stored sealed at room temperature until required for *in vitro* digestion and *in vitro* fermentation experiments.

6.3.4 Polysaccharide molecular weight

The average molecular weight of PPE was determined using a modified high performance size exclusion chromatography method from Gómez-Ordónez *et al.* (Gomez-Ordonez et al., 2012). In brief, separation was performed on a PL aquagel-oh mixed-H 8µm SEC analytical column (7.5 x 300 mm i.d) with isocratic elution at 50°C and a flow rate of 0.6 mL/min and a run time of 31 mins, using a 50mM ammonium formate mobile phase and a ten-point pullulan standard curve (0.34 to 708 kDa).

6.3.5 Polysaccharide *in vitro* digestion

An *in vitro* simulated digestion was completed on each seaweed polysaccharide following the method of Minekus *et al.* (Minekus et al., 2014), with oral, gastric, and intestinal phases of digestion. All simulated fluids were incubated at 37°C before use and all digestion stages were incubated at 37°C in an orbital shaker (160 rpm). The oral suspension was incubated for 2 mins, while both the gastric and intestinal suspensions were incubated for 2 hrs. The intestinal phase digesta was

dialysed for 24 hrs using 1kDa dialysis tubing to mimic intestinal absorption (Spectrum Labs, USA) and the retentate was freeze dried (Labconco, USA) to obtain powders before batch culture fermentation.

6.3.6 Batch culture fermentation - preparation of human faecal inoculum and basal media

A 20% faecal slurry was prepared following the methods of O'Donnell *et al.* (O'Donnell et al., 2016). The resulting faecal bacteria suspension was amended with sterile glycerol to a final concentration of 25% (v/v) and stored frozen at -80°C until use. Nutrient basal medium stock solution was prepared using methodology of Fooks and Gibson (Fooks and Gibson, 2003).

6.3.7 Batch culture fermentation

Digested, freeze-dried, seaweed polysaccharide powders underwent *in vitro* batch culture faecal fermentation for 24 hrs using the MicroMatrix bioreactor (Applikon Biotechnology, The Netherlands) (O'Donnell et al., 2018). Synergy 1 (Beneo, Germany) was used as a positive control (n=8) and cellulose was used as a negative control (n = 8). Seaweed polysaccharides were fermented in quadruplicate. Vessels were inoculated with 5 % (v/v) faecal slurry and the designated carbon source was added at 1 % (w/v). The MicroMatrix bioreactor was operated using MicroMatrix Human Machine Interface software (Applikon Biotechnology, Delft, The Netherlands), which controlled orbiter speed (250 rpm), pH 6.8 (liquid addition of 4M NaOH), temperature (37°C), and dissolved oxygen (individually controlled gas addition of N_2 and CO_2). Samples were obtained at t = 0 and 24 hrs and

immediately centrifuged at 21,382 RCF to provide a pellet for DNA extraction and a supernatant for SCFA analysis. All samples were stored at -80°C until analysis.

6.3.8 DNA extraction

Genomic DNA was extracted from samples after 0 hrs and 24 hrs fermentation using the PowerFecal DNA extraction kit (Mo Bio Laboratories, Carlsbad, USA) according to manufactures' instructions. The bead beating step was completed using the Mo Bio vortex adapter.

6.3.9 qPCR

Total bacteria, *Lactobacillus* spp., and *Bifidobacterium* spp. were quantified using qPCR. Primer sequences used for qPCR (Target: Forward primer 5'-3'; Reverse primer 5'-3'; Size bp; Tm °C): Total Bacteria (Eubacterial): ACTCCTACGGGAGGCAGCAG; ATTACCGCGGCTGCTGG; 200 bp; 60°C. *Lactobacillus* genus: GCAGCAGTAGGGAATCTTCCA; GCATTYCACCGCTACACATG; 349 bp; 60°C. *Bifidobacterium* genus: CTCCTGGAAACGGGTGGT; GCTGCCTCCCGTAGGAGT; 203 bp; 60°C. A standard curve of 10^9-10^3 CFU/ml was prepared in duplicate for each plate. A PCR master mix was prepared with the forward and reverse primers, SYBR® FAST pPCR Master Mix (KAPA Biosystems, USA), and PCR water. 1 µl of sample DNA or PCR water (negative control) was added to 9 µl master mix per well (reaction volume = 10 µl) and ran in duplicate on two plates (n = 4). The Lightcycler® 480 Instrument II (Roche, Switzerland) was used with the following PCR conditions: denaturation = 1 cycle; amplification = 40 cycles; melting = 1 cycle; cooling = 1 cycle. Target temperature was 95°C with a hold time of 3 min and a ramp rate of 4.4°C/sec.

6.3.10 16S rRNA amplification and MiSeq sequencing

Illumina MiSeq sequencing library preparation was completed following the 16S metagenomic sequencing library protocol (Illumina, USA) and as described by Fouhy *et al*. (Fouhy et al., 2015). Amplicon PCR: Genomic DNA was amplified using primers specific to the V3-V4 hypervariable region of the 16S ribosomal RNA gene to create a 460bp amplicon. These primers also incorporated the Illumina overhang adaptor (Forward primer 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

reverse primer 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Each PCR reaction contained 2.5 µL template DNA, 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 12.5 µL 2X Kapa HiFi Hotstart ready mix (KAPA Biosystems, USA) giving a total of 25µL in the final reaction volume. PCR amplification was carried out using the Applied Biosystems 2720 thermal cycler (Life Technologies, USA) with the following parameters: heated lid 110°, 95°C for 3 mins; then 25 cycles of: 95°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs; hold at 72°C for 5 mins; hold at 4°C. Successful PCR products were cleaned using Agentcourt AMPure XP kit (Beckman Coulter Genomics, UK). A second PCR reaction was completed to attach the Illumina sequencing adapters onto the amplicons using the Nextera XT Index kit (Illumina, USA). The DNA concentration of each sample was determined using the Qubit High Sensitivity DNA kit and the Qubit 3 Fluorometer (Invitrogen, USA). DNA samples were then pooled as an equimolar mix and sequenced on the MiSeq sequencing platform at Teagasc, Moorepark,

Ireland following standard Illumina sequencing protocols for the 2 × 250 cycle V3 Kit.

6.3.11 Bioinformatics

Two hundred and fifty base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (Caporaso et al., 2010). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123.

6.3.12 Short chain fatty acid and branched chain fatty acid analysis

GC-MS analysis was carried out using a modified protocol described by Garcia-Villalba *et al.* (Garcia-Villalba et al., 2012). In brief, phosphoric acid was added to samples to a final concentration of 0.5 % (v/v) prior to ethyl acetate extraction (1:1 v/v). 180µl organic phase was added to a GC vial alongside 20 µl of 4-methyl valerate internal standard. A standard curve of 10, 20, 50, 100, 500, 1000, 5000, 10000, 50000, and 100,000 µM SCFA mix containing acetic acid, propionic acid, n-butyric acid, i-butyric acid, valeric acid, i-valeric acid, and hexanoic acid was ran within every sample batch. Quality control consisted of two 50 µM and two 100 µM standard mixes every sixteen vials and ethyl acetate blanks every six vials, and

between each standard vial/QC to prevent carryover. The GC-MS system consisted of an Agilent 6890N (Agilent Technologies, USA), equipped with an Agilent 7683 AutoSampler and 7683B injector, coupled to an Agilent 5973 inert mass selective detector. Agilent MassHunter GC/MS Acquisition software was used. The GC was fitted with a DB-WAXetr capillary column (30m length, 0.25mm i.d, 0.25µm film thickness), with helium used as the carrier gas (1.2mL/min). Injections were made in splitless mode with an injection volume of 1µL (10 µL syringe) and an injection temperature of 250°C. The syringe undertook four pre-washes and four post-washes in hexane. The initial column temperature was 90°C, and ramped to 150°C at 15°C/min, then to 170°C at 5°C/min, then to 230°C at 20°C/min, where it was maintained for 2 mins. Total run time was 14 mins. Solvent delay was 2.5 mins. The detector was operated in electron impact ionisation mode.

6.3.13 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics (version 24) and GraphPad Prism 8 software. Graphs were made in GraphPad Prism 8 software. To determine significant effects of each treatment on bacterial enumeration (qPCR) and SCFA concentration (GC-MS), statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (*q* value) with a significance level of ≤ 0.05.

Statistical analysis of 16S rRNA sequencing data was carried out using Calypso online software (version 8.68) (Zakrzewski et al., 2017). Data were normalized using cumulative sum scaling and $\log_2$ transformed to account for the non-normal

distribution of sequencing data (Paulson et al., 2013). Up to 20,000 taxa with > 0.01% abundance were used in the analysis. Chloroplasts and cyanobacteria were removed from the analysis.

Alpha diversity (non-normalised operational taxonomic unit data) was determined using rarefied Chao1, Evenness, Shannon and Simpson indices. Statistical significance was determined using a non-parametric Kruskal-Wallis test (*p* value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (*q* value) with a significance level of ≤ 0.05. A Permutational multivariate analysis of variance (PerMANOVA) was used to determine statistical differences in Bray-Curtis dissimilarity distance matrices of beta diversity.

Statistical significance of mean bacterial relative abundances, compared to cellulose, at the phylum, family, and genus level after 0, 10, and 24 hrs fermentation was determined using a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons and a significance level of $p \leq 0.05$. A feature selection analysis (linear discriminant analysis effect size - LEfSe) was used to highlight discriminate taxa between fermentation substrates and cellulose at 0 and 24 hrs (Segata et al., 2011).

6.4 Results

6.4.1 Seaweed polysaccharide molecular weight

The average molecular weight of the seaweed polysaccharides used in this study is presented in **Table 6-1**. Both sources of alginate had a similar molecular weight, however, the molecular weight of the two fucoidan substrates differed almost 10-

fold. It was also observed that Alginate-Fv had a minor peak of 7.2 kDa; Ulvan had minor peaks at 195 kDa and 4.5 kDa; and Xylan-Pp had a molecular weight distribution of 21 - 650 kDa.

6.4.2 qPCR

Total bacteria was significantly higher in vessels fermented in the presence of Fucoidan An (p = 0.0169, q = 0.0438), Laminarin Ld (p = 0.0008, q = 0.0031), Xylan Pp (p = 0.0219, q = 0.0438), and Synergy 1 (p = 0.0001, q = 0.0012) when compared to cellulose after 24 hrs (**Figure 6-1**).

There was no statistical difference in the number of *Lactobacillus* spp. in vessels fermented with any seaweed polysaccharide substrate when compared to cellulose after 24 hrs fermentation, although fermentation in the presence of Synergy 1 resulted in significantly higher *Lactobacillus* populations when compared to cellulose (p = 0.0045, q = 0.0359) (**Figure 6-2**). The number of *Bifidobacterium* spp. were significantly higher following fermentation in the presence of Xylan-Pp (p = 0.0025, q = 0.0101) and Synergy 1 (p = 0.0006, q = 0.0046) when compared to cellulose after 24 hrs (**Figure 6-3**). There was no statistical difference in the number of *Bifidobacterium* spp. detected in vessels fermented with Xylan-Pp and Synergy 1 ($p > 0.5$, $q > 0.5$).

6.4.3 16S rRNA amplicon sequencing: Effect of seaweed polysaccharides on the bacterial diversity of the faecal microbiota

There was no statistical difference in the alpha diversity metrics of Evenness, Chao 1 richness, Shannon diversity index, and Simpson's diversity index in vessels fermented with any test substrate when compared to cellulose after 24 hrs

fermentation (q > 0.5). The Bray-Curtis dissimilarity index of beta diversity was significantly different between vessels fermented with Synergy 1 when compared to cellulose after 24 hrs (p = 0.012), but not in vessels fermented with seaweed polysaccharides (Alginate-Lj, p = 0.265; Alginate-Fv, p = 0.0766; Fucoidan-Fv, p = 0.0846; Fucoidan-An, p = 0.297; Laminarin-Ld, p = 0.0813; Ulvan, p = 0.486; Xylan-Pp, p = 0.244).

6.4.4 16S rRNA amplicon sequencing: Effect of seaweed polysaccharides on the microbial abundance of the faecal microbiota

The mean relative abundance of bacterial taxa in vessels fermented in the presence of cellulose, Alginate-Lj, Alginate-Fv, Fucoidan-Fv, Fucoidan-An, Laminarin-Ld, Ulvan, Xylan-Pp, and Synergy 1 are shown at the phylum (**Figure 6-4**), family (**Figure 6-5**), and genus (**Figure 6-6** and **Figure 6-7**) levels of taxonomy.

After 24 hrs fermentation in the presence of Alginate-Lj, there was no statistical difference in the relative abundance of bacterial taxa at the phylum, family, and genus levels when compared to cellulose (p > 0.05); however, fermentation in the presence of Alginate-Fv resulted in a significant higher relative abundance of the family *Bacteroidaceae* (p = 0.007) and the genus *Bacteroides* (p = 0.023) when compared to cellulose (**Table 6-2**) after 24 hrs. There was no statistical difference in the relative abundance of *Bacteroides* in vessels fermented with Alginate-Fv when compared to Synergy 1 (p = 0.122). LEfSe analysis indicated that the genera *Acidaminococcus*, *Akkermanisa*, *Alistipes*, *Bacteroides*, *Bilophila*, *Christensenellaceae R7* group, *Clostridium sensu stricto 1*, *Clostridium sensu stricto 13*, *Dielma*, *Enterococcus*, *Erysipelatoclostridium*, *Haemophilus*, *Parabacteroides*,

Peptoclostridium, *Phascolarctobacterium*, *Sutterella*, *Veillonella*, and an uncultured bacterium were discriminative bacteria of fermentation in the presence of Alginate-Fv when compared to cellulose (**Figure 6-8**). While *Holdemania* was a discriminative genus of Alginate-Lj fermentation when compared to cellulose (**Figure 6-9**).

Fermentation in the presence of Fucoidan-Fv and fermentation in the presence of Fucoidan-An resulted in no statistically significant differences in the relative abundance of bacterial taxa at the phylum, family, and genus levels after 24 hrs when compared to cellulose ($p > 0.05$). LEfSe analysis indicated that genera of the *Eubacterium coprostanoligenes* group, *Eubacterium hallii* group, *Dorea*, *Parasutterella*, *Pseudobutyrivibrio*, *Roseburia*, and *Ruminococcaceae UCG013* were discriminative genera of fermentation in the presence of Fucoidan-Fv (**Figure 6-10**), while *Anaerotruncus*, *Barnesiella*, and *Peptococcus* were discriminative genera of fermentation in the presence of Fucoidan-An (**Figure 6-11**).

Fermentation in the presence of Laminarin-Ld resulted in a significantly higher relative abundance of the family *Lachnospiraceae* ($p = 0.001$) and the genera *Erysipelatoclostridium* ($p = 0.001$) and *Catenibacterium* ($p = 0.001$) after 24 hrs when compared to cellulose (**Table 6-3**). Fermentation in the presence of Laminarin-Ld resulted in a significantly lower relative abundance of the families *Thermoanaerobacteraceae* ($p = 0.038$), *Family XI* ($p = 0.015$), and *Erysipelotrichaceae* ($p = 0.041$); and the genera *Turicibacter* ($p = 0.028$), *Howardella* ($p = 0.020$), and *Gelria* ($p = 0.019$) when compared to cellulose after 24 hrs. LEfSe analysis indicated that *Alistipes*, *Catenibacterium*, *Erysipelotrichaceae UCG003*,

Erysipelatoclostridium, and *Lachnospiraceae UCG004* were discriminative genera of fermentation in the presence of Laminarin-Ld (**Figure 6-12**).

Fermentation in the presence of Ulvan resulted in a significantly lower relative abundance of the families *Pasteurellaceae* (p = 0.036) and *Fusobacteriaceae* (p = 0.036) when compared to cellulose after 24 hrs; however, no statistically significant differences were observed at the genus level (**Table 6-4**). LEfSe analysis indicated that *Parasutterella* and *Peptococcus* were discriminative genera of fermentation in the presence of Ulvan when compared to cellulose (**Figure 6-13**).

Fermentation in the presence of Xylan-Pp resulted in a significantly lower relative abundance of the genera *Gelria* (p = 0.042) and *Paraprevotella* (p = 0.016) when compared to cellulose after 24 hrs. While a significant higher abundance of the genus *Bifidobacterium* (p = 0.0002), the family *Bifidobacteriaceae* (p = 0.00017), and the phylum Actinobacteria (p = 0.00173) were observed in vessels fermented in the presence of Xylan-Pp when compared to cellulose (**Table 6-5**). There was no statistical difference in the relative abundance of *Bifidobacterium* in vessels fermented in the presence of Xylan-Pp when compared to Synergy 1 after 24 hrs (p > 0.5). LEfSe analysis demonstrated that *Lachnoclostridium 5*, *Bifidobacterium*, and *Blautia* were discriminative genera of Xylan-Pp fermentation when compared to cellulose after 24 hrs (**Figure 6-14**).

Fermentation in the presence of Synergy 1 resulted in several significant differences in the mean relative abundance of bacterial taxa when compared to cellulose after 24 hrs (**Table 6-6**). This included significantly higher relative abundances of the genera *Erysipelatoclostridium* (p < 0.0001), *Ruminococcus 1* (p =

0.039), *Streptococcus* (p = 0.024), *Leuconostoc* (p = 0.009), *Catenibacterium* (p < 0.0001), and *Bifidobacterium* (p < 0.0001), alongside a significantly lower relative abundances of *Paraprevotella* (p = 0.001), *Howardella* (p = 0.031), and *Desulfovibrio* (p = 0.025). LEfSe analysis showed that *Bacteroides*, *Bifidobacterium*, *Blautia*, *Catenibacterium*, *Clostridium sensu stricto 1*, *Collinsella*, *Erysipelatoclostridium*, *Lachnospiraceae UCG004*, *Leuconostoc*, *Streptococcus*, *Roseburia*, *Ruminococcus 1*, and an uncultured bacterium were discriminative genera of fermentation in the presence of Synergy 1 when compared to cellulose (**Figure 6-15**).

6.4.5 Effect of seaweed polysaccharides on short chain fatty acid production

There was no statistical difference between the concentrations of acetic, propionic, butyric, valeric, hexanoic, and total SCFA for any fermentation substrate when compared to cellulose at 0 hrs (**Figure 6-16**). After 24 hrs fermentation, the concentration of acetic acid was significantly higher in vessels fermented with Ulvan (p = 0.001) and Xylan-Pp (p = < 0.0001) when compared to cellulose (**Figure 6-16**). These data were not statistically different to Synergy 1 (p > 0.5), which demonstrated a trend towards higher concentrations of acetic acid when compared to cellulose (p = 0.054). Propionic acid concentration was significantly higher in vessels fermented in the presence of Xylan-Pp (p < 0.0001) and Laminarin-Ld (p = 0.017) when compared to cellulose after 24 hrs (**Figure 6-16**). There was no statistical difference between propionic acid concentrations in vessels fermented with Xylan-Pp and Laminarin-Ld when compared to Synergy 1 (p > 0.5) (**Figure 6-16**). Fermentation in the presence of Synergy 1 also resulted in

significantly higher concentrations of propionic acid when compared to cellulose after 24 hrs ($p < 0.001$) (**Figure 6-16**). The concentration of butyric acid was also significantly higher in vessels fermented in the presence of Alginate-Fv ($p = 0.034$) and Synergy 1 ($p < 0.001$) when compared to cellulose after 24 hrs (**Figure 6-16**). There was no statistical difference between the butyric acid concentrations in vessels fermented in the presence of Alginate-Fv when compared to Synergy 1 ($p > 0.5$) after 24 hrs. The concentration of valeric acid was significantly higher in vessels fermented in the presence of Xylan-Pp when compared to cellulose after 24 hrs ($p = 0.003$) (**Figure 6-16**). This was not statistically different to Synergy 1 ($p > 0.5$). The concentration of hexanoic acid was significantly lower in vessels fermented in the presence of Fucoidan-Fv when compared to cellulose after 24 hrs ($p < 0.001$), but not statistically different to Synergy 1 ($p > 0.5$) (**Figure 6-16**). Total SCFA concentrations were significantly higher in vessels fermented in the presence of Xlyan-Pp ($p < 0.001$) and Synergy 1 ($p < 0.001$) when compared to cellulose (**Figure 6-16**). Total SCFA concentration was not statistically different between vessels fermented with Xylan-Pp compared to Synergy 1 ($p > 0.5$).

6.4.6 Effect of seaweed polysaccharides on branched chain fatty acid production

There was no statistical difference between the concentration of isobutyric, isovaleric, and total branched chain fatty acids (BCFA) in vessels fermented in the presence of any test substrate when compared to cellulose at 0 hrs (**Figure 6-17**). After 24 hrs fermentation, isobutyric acid concentrations were significantly higher in vessels fermented in the presence of Alginate-Fv ($p = 0.001$), Alginate-Lj ($p =$

0.005), and Xylan-Pp (p = 0.043) when compared to cellulose, but not when compared to Synergy 1 (p > 0.5) (**Figure 6-17**). Isovaleric acid concentrations were significantly higher in vessels fermented in the presence of Alginate-Lj (p = 0.008) and Fucoidan-An (p = 0.009) when compared to cellulose (**Figure 6-17**); while these data were not statistically different to Synergy 1 (p > 0.1). Total BCFA were significantly higher in vessels fermented in the presence of Alginate-Fv (p = 0.001) and Alginate-Lj (p = 0.003) when compared to cellulose after 24 hrs, but not when compared to Synergy 1 (p = 0.063 and p = 0.145, respectively) (**Figure 6-17**).

6.5 Discussion

The data obtained in this study indicate that xylan, laminarin, and alginate seaweed polysaccharides were fermented by gut microbial populations owing to substantively higher concentrations of SCFA when compared to cellulose. 16S rRNA amplicon sequencing data showed a statistically significant higher relative abundance of *Bifidobacterium* for xylan, *Bacteroides* for alginate, and *Lachnospiraceae* for laminarin, when compared to cellulose. No significant changes in bacterial genera were noted for fucoidan and ulvan substrates there was no statistical difference in alpha and beta indices of phylogenetic diversity when compared to cellulose.

6.5.1.1 Brown seaweed polysaccharides – Fucoidan, Alginate & Laminarin

Fucoidan treatment had no statistical impact on total bacteria or the traditional prebiotic markers of *Lactobacillus* spp. or *Bifidobacterium* spp. (qPCR), or in bacterial mean relative abundance (16S rRNA sequencing). This contrasts with previous *in vitro* studies which reported that *Laminaria japonica* derived fucoidan

increased *Lactobacillus* and *Bifidobacterium* (Kong et al., 2016), whilst *Ascophyllum nodosum* derived fucoidan resulted in elevated *Bacteroides*, *Phascolarctobacterium*, *Oscillospira*, and *Faecalibacterium* (Chen et al., 2018). Evidence from animal studies has shown an impact of fucoidan on the caecal microbiota as noted by increased caecal *Lactobacillus* in healthy mice (Shang et al., 2016); and increased caecal populations of *Akkermansia*, *Alloprevotella*, *Blautia*, and *Bacteroides* in mice with metabolic syndrome (Shang et al., 2017). Whilst the current study did not see any significant changes associated with fucoidan treatment in relation to phylum, family or genus, the discriminatory analysis identified fucoidan treatment to be associated with genera that express α-fucosidases including *Barnesiella* spp. (Fucoidan-An), *Roseburia* (Fucoidan-Fv), and *Ruminococcaceae UCG013*. The current study showed that fucoidan from two different seaweed species had no significant impact on SCFA production (aside of minor changes to hexanoic acid) indicating limited fermentability of fucoidan, which has also been previously reported in rats (An et al., 2013). The lack of any significant changes in microbiota composition alongside no differences in SCFA concentration when compared to cellulose would collectively indicate a lack of fucoidan utilization by the microbiota. Nevertheless, this could potentially be explained by the limitations of establishing a representative microbiota in an *in vitro* experimental setup and should be investigated further in *in vivo* experiments.

Neither of the two alginate substrates (from Alginate-Fv - 124kDa; Alginate-Lj - 177kDa) exerted a bifidogenic effect. This is in agreement with one previous *in vitro* fermentation study with an alginate of 212kDa (Ramnani et al., 2012). Alginate consumption, however, has been shown to exert bifidogenic effects in

mice (3.5 kDa) (Wang et al., 2006) and in humans (30kDa) (Terada et al., 1995). The noted disparities may be explained by the differences in structural composition of alginate sources in relation to the ratio of 1-4-linked α-L-guluronic (G) and β-D-mannuronic acid (M), which is an important factor to consider in relation to alginate utilization and bioactivity (Houghton et al., 2015).

Alginate-Fv fermentation resulted in a significantly higher relative abundance of the genus *Bacteroides* in comparison to cellulose. This corroborates evidence from a previous *in vitro* faecal fermentation with an alginate substrate (Bai et al., 2017), as well as a rodent study which demonstrated increased caecal *Bacteroides* spp. after alginate supplementation (An et al., 2013). *Bacteroides* are primary degraders and have been reported to degrade alginate owing to the expression of alginate lyase enzymes (Li et al., 2017; Maruyama et al., 2015; Mathieu et al., 2018, 2016; Thomas et al., 2012). It could be postulated that degradation of alginate by *Bacteroides* may have contributed to the stimulation of *Phascolarctobacterium Parabacteroides* and *Alistipes* genera which were associated with alginate treatment in the discriminatory analysis and should be explored further. Bacterial mean relative abundance and SCFA concentration in vessels treated with Alginate-Lj was not statistically different to vessels treated with cellulose. In contrast, Alginate-Fv fermentation resulted in a significantly higher concentration of butyric acid after 24 hrs indicating that Alginate-Fv was fermented by bacterial populations.

Given the disparities in molecular weight and structure of alginates used between the present study and previous studies, there is scope to investigate how the

structure and molecular weight of alginate impacts its fermentability. Further, the current study indicates that specific alginates may increase concentrations of colonic butyrate and increase the relative abundance of genera within the CAZyme-rich Bacteroidetes phylum. In terms of health benefits, this may be associated with gut microbiota-mediated regulation of glucose and lipid metabolism, considering that alginate supplementation is reported to improve weight loss and reduce cholesterol and glucose uptake (Chater et al., 2015; Georg Jensen et al., 2012b, 2012a; Lange et al., 2015; Paxman et al., 2008).

Laminarin-Ld did not stimulate populations of *Bifidobacterium*, *Lactobacillus*, or *Bacteroides* in comparison to cellulose. The noted higher relative abundance of the family *Lachnospiraceae* following Laminarin-Ld treatment corroborates similar observations noted in a previous *in vitro* fermentation study using a *Laminaria digitata* polysaccharide-rich extract (Strain et al., 2019), as well as an *in vivo* study in rodents fed 2% (w/w) laminarin (An et al., 2013). High fibre diets have been associated with higher faecal *Lachnospiraceae* populations, which has been proposed to be attributable to the a myriad of polysaccharide utilisation loci (PUL) which express CAZymes that degrade complex plant carbohydrates to produce SCFA such as butyrate (Biddle et al., 2013; Bishehsari et al., 2018; Tidjani Alou et al., 2016). Future studies should undertake genomic or proteomic approaches to determine the presence, expression and activity of β-glucanases or β-glucosidases in *Lachnospiraceae* following laminarin treatment.

Laminarin-Ld fermentation resulted in a significant reduction in *Turicibacter* and *Gelria* when compared to cellulose. The impact of such changes is difficult to

determine but it must be noted that *Turicibacter* are pathobionts associated with gut inflammation (Goodrich et al., 2016; Rausch et al., 2015), while *Gelria* are methanogens (Mancabelli et al., 2017). Therefore, the capacity of laminarin to modulate the gut microbiota to potentially reduce gut inflammation and methane production could be beneficial to gastrointestinal health if confirmed *in vivo*.

Laminarin-Ld treatment resulted in a significantly higher concentration of propionic acid after 24 hrs when compared to cellulose. This is in agreement with previous studies and suggests that the beta glucan, Laminarin (1,3- 1,6-linked β-D-glucose), is fermentable (An et al., 2013; Devillé et al., 2007; Michel et al., 1996; Seong et al., 2019). It is understood that propionate can modulate hepatic and peripheral metabolism and stimulate anorexigenic signaling via free fatty acid receptor activation to regulate appetite and energy homeostasis (Byrne et al., 2015; Rowland et al., 2018). Therefore, an *in vivo* investigation into the effects of dietary Laminarin intake may be warranted, considering the evidence that increased colonic propionate has been shown to limit weight gain in overweight humans (Chambers et al., 2015).

6.5.1.2 Green seaweed polysaccharide - Ulvan

Ulvan fermentation did not stimulate *Lactobacillus* or *Bifidobacterium* populations when compared to cellulose, which is in agreement with a previous *in vitro* faecal fermentation experiment with an *Enteromorpha prolifera* polysaccharide extract (Kong et al., 2016). Data from the present study contrast with another *in vitro* fermentation study which observed an enrichment of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* in ulvan-treated vessels (Seong et al., 2019).

While murine studies have demonstrated that a polysaccharides from *Enteromorpha* spp. significantly increased faecal Actinobacteria (Ren et al., 2017) and caecal *Bifidobacterium*, *Lactobacillus*, and *Akkermansia muciniphila* (Shang et al., 2018).

Ulvan-treated vessels had a significantly higher concentration of acetic acid when compared to cellulose after 24 h. Although this is in agreement with another *in vitro* fermentation study (Seong et al., 2019), it is in contrast with early studies which indicated that ulvan was poorly fermented (Andrieux et al., 1998; Bobin-Dubigeon et al., 1997). One explanation for the lack of corroborating evidence regarding the fermentability of ulvan may be owing to its highly heterogenous structure (repeating α-L-rhamnose-3-sulfate-1,4-β-D-glucuronic acid, α-L-rhamnose-3-sulfate-1,4-α-D-iduronic acid, and α-L-Rhamnose-3-sulfate-1,4-β-D-xylose) (Michel and Czjzek, 2013). Moreover, very few ulvan-specific hydrolytic enzymes, such as ulvan lyase, have been identified (Konasani et al., 2018; Kopel et al., 2016), while human gut bacteria may need to express a similar enzymatic cascade of polysaccharide lyases, sulfatases and glycoside hydrolases, as described in the marine bacterium *Formosa agariphila* (Reisky et al., 2019). It has also been suggested that the fermentability of ulvan is related to the degree of sulphation and molecular weight (Kong et al., 2016). Given that some 40% of the sulphate content of ulvan is dissimulated to sulphide by sulphate reducing bacteria such as *Desulfovibrio* (Durand et al., 1997; Figliuolo et al., 2017), determination of potentially deleterious metabolites produced following the consumption of ulvan may also be warranted. A greater understanding of how ulvan structure affects its

fermentability by gut bacterial populations is needed, given the disparity between data from the current study and other *in vitro* and *in vivo* studies.

6.5.1.3 Red seaweed polysaccharide - Xylan

Xylan-Pp treatment resulted in significantly higher abundance of *Bifidobacterium* spp. when compared to cellulose, based on qPCR and 16S rRNA amplicon sequencing data, and this bifidogenic effect was not statistically different to the Synergy 1 positive control. Furthermore, LEfSe analysis indicated that *Bifidobacterium* was a discriminative genus of Xylan-Pp fermentation. Given that a bifidogenic effect is a seminal characteristic of a potential prebiotic, there is scope to examine the impact of xylan from *Palmaria palmata* on health-related endpoints *in vivo*. This aligns with the bifidogenic effect of xylans and xylo-oligosaccharides extracted from terrestrial plants which have also been shown to improve plasma lipid profile and immune function in healthy adults (Childs et al., 2014; Finegold et al., 2014; Lecerf et al., 2012; Moniz et al., 2016; Yang et al., 2015).

The genera *Blautia* and *Lachnoclostridium 5* were also discriminative genera of Xylan-Pp fermentation. *Lachnoclostridium*-derived butyrate has been implicated in the promotion of intestinal immune homeostasis (Martin-Gallausiaux et al., 2018). Furthermore, *Blautia* can ferment dietary complex polysaccharides to produce acetate, propionate and butyrate SCFA and are a widely abundant genus of the human gut microbiota deemed beneficial to health (Park et al., 2013; Rojo et al., 2017; Tidjani Alou et al., 2016). A significant increase in *Blautia* has been previously reported in pre-type-two-diabetic individuals following an eight week intervention

with 2 g/day xylo-oligosaccharides derived from terrestrial plants (Yang et al., 2015). Similar to Laminarin-Ld, Xylan-Pp fermentation resulted in a significantly lower abundance of the potentially putrefactive *Gelria* (Mancabelli et al., 2017) however, *in vivo* evidence is needed to ascertain whether dietary intakes of xylan can inhibit the growth of potential gut pathogens.

Xylan-Pp treatment resulted in a significantly higher concentrations of acetic acid, propionic acid, valeric acid, and total SCFA in comparison to cellulose after 24 hrs, which aligns with the noted changes in SCFA producing genera and provides a clear indication that this substrate was fermentable. These data are in agreement with the only previous *in vitro* fermentation study of xylan extracted from *Palmaria palmata*, which described a rapid fermentation of xylan with the production of SCFA after six hrs (Lahaye et al., 1993). This would suggest that a repertoire of xylanases and xylosidases with specificity for the 1,3-1,4-β-D-xylose of seaweed-derived xylans exists within the human gut bacteriome, akin to terrestrial plant-derived xylans (Caroline Mirande et al., 2010; C. Mirande et al., 2010). An example of the latter includes *Bifidobacterium* spp., which are reported to transcribe enzymes which hydrolyse xylose from complex glycans (Turroni et al., 2016). Given the enrichment of health-associated bacteria, including *Bifidobacterium* spp., alongside higher concentrations of SCFA by Xylan-Pp when compared to cellulose in the present study, xylans and xylo-oligosaccharides obtained from the red seaweed *Palmaria palmata* warrant further evaluation for prebiotic efficacy. Future investigations should include *in vivo* studies to understand what impact dietary intake of xylan from *Palmaria palmata* has on host health, and to compare their effect with that of terrestrial plant-derived xylans.

The use of a pooled faecal inoculum in this study was intended to provide a reproducible inoculant for each fermentation vessel treatment to reduce the effect of inter-individual variation in microbiota composition between donors (O'Donnell et al., 2016). One limitation of using a batch culture *in vitro* colonic fermentation model compared to the *in vivo* situation is that *certain* bacteria are enriched or depleted, owing to inexact replication of the *in vivo* colonic environment (Verhoeckx K, 2015). For example, the lack of mucus within the fermentation system could impact the growth of mucin degraders such as *Akkermansia* spp.. An alternative screening approach could include dynamic *in vitro* models (Van den Abbeele et al., 2010), but this does not replicate the *in vivo* gut, either.

Fermentation in the presence of Alginate-Lj, Alginate-Fv, Fucoidan-An, and Xylan substrates also resulted in higher BCFA concentrations compared to cellulose. This suggests that proteolytic fermentation occurred. Therefore, understanding how these substrates impact concentrations of potentially genotoxic compounds in the gut, such as ammonia and *p*-Cresol, is needed when considering future *in vivo* studies (Diether and Willing, 2019).

6.6 Conclusion

The data obtained from this study provide evidence that the seaweed-derived polysaccharides, namely alginate, laminarin, and xylan are microbiota-accessible carbohydrates for bacterial fermentation and can modulate the composition and metabolic activity of the human faecal microbiota *in vitro*. Further investigation is required to understand the fermentability of fucoidan and ulvan complex

polysaccharides. The most promising prebiotic candidate is xylan obtained from *Palmaria palmata*, owing to its bifidogenic effect and increased production of health associated SCFA. Future directions should explore oligosaccharides obtained from seaweed polysaccharides, and to explore how the structure of seaweed polysaccharides impact saccharolytic fermentation by the gut microbiota. *In vivo* studies are needed to understand the impact of seaweed polysaccharides on gut microbiota composition and metabolic activity, and to understand whether such modulation can confer a benefit to host health.

6.7 References

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6.8 Figures

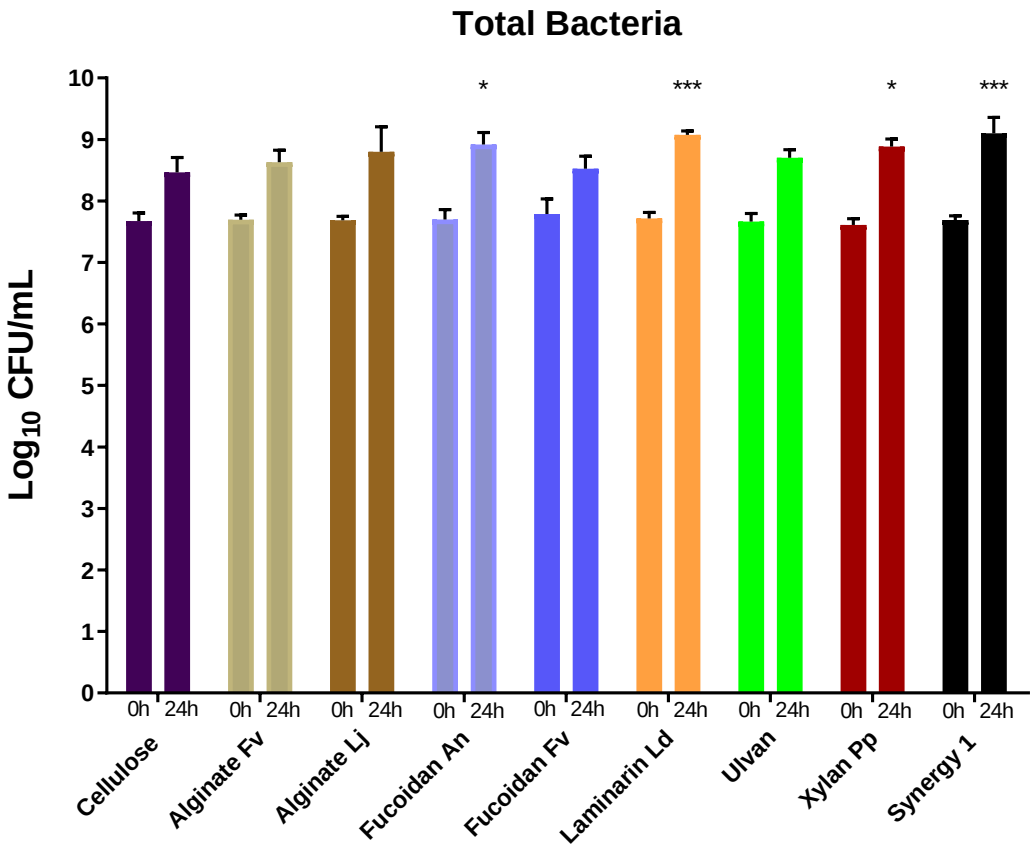

Figure 6-1. qPCR was used to quantify total bacteria from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, or Synergy 1 substrates. Samples were taken at 0 and 24 hrs fermentation. Data plots represent the mean + standard deviation of the mean. Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

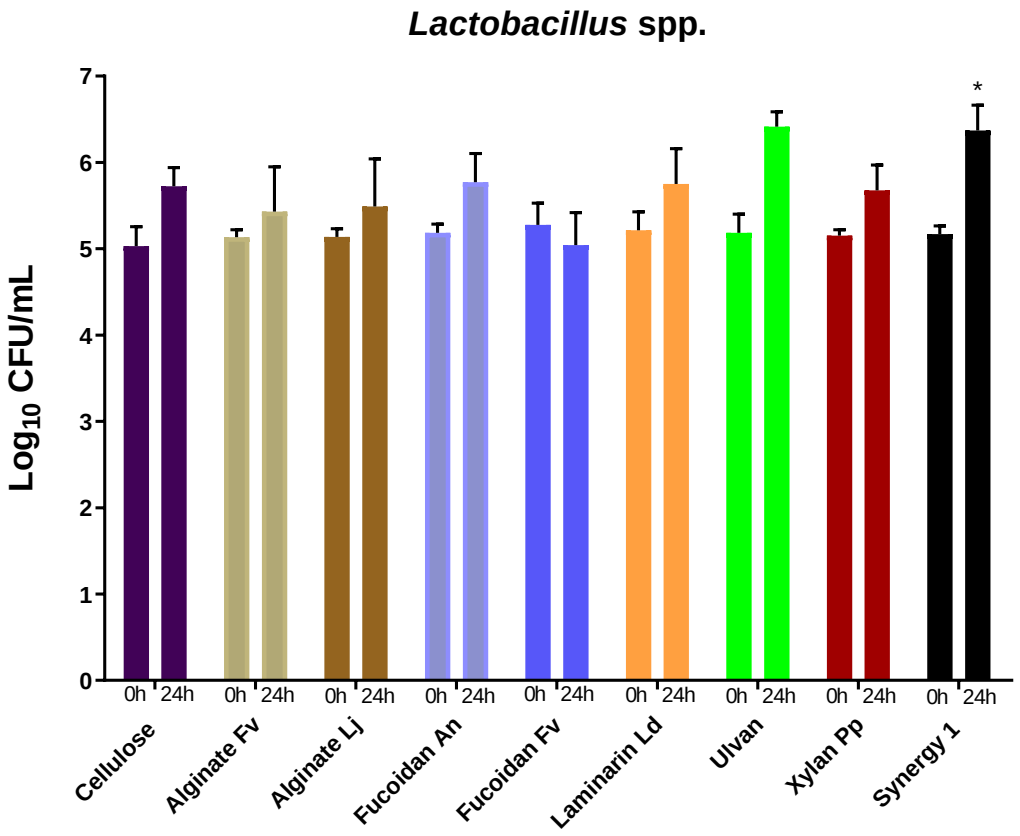

Figure 6-2. qPCR was used to quantify *Lactobacillus* spp. from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, or Synergy 1 substrates. Samples were taken at 0 and 24 hrs fermentation. Data plots represent the mean + standard deviation of the mean. Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

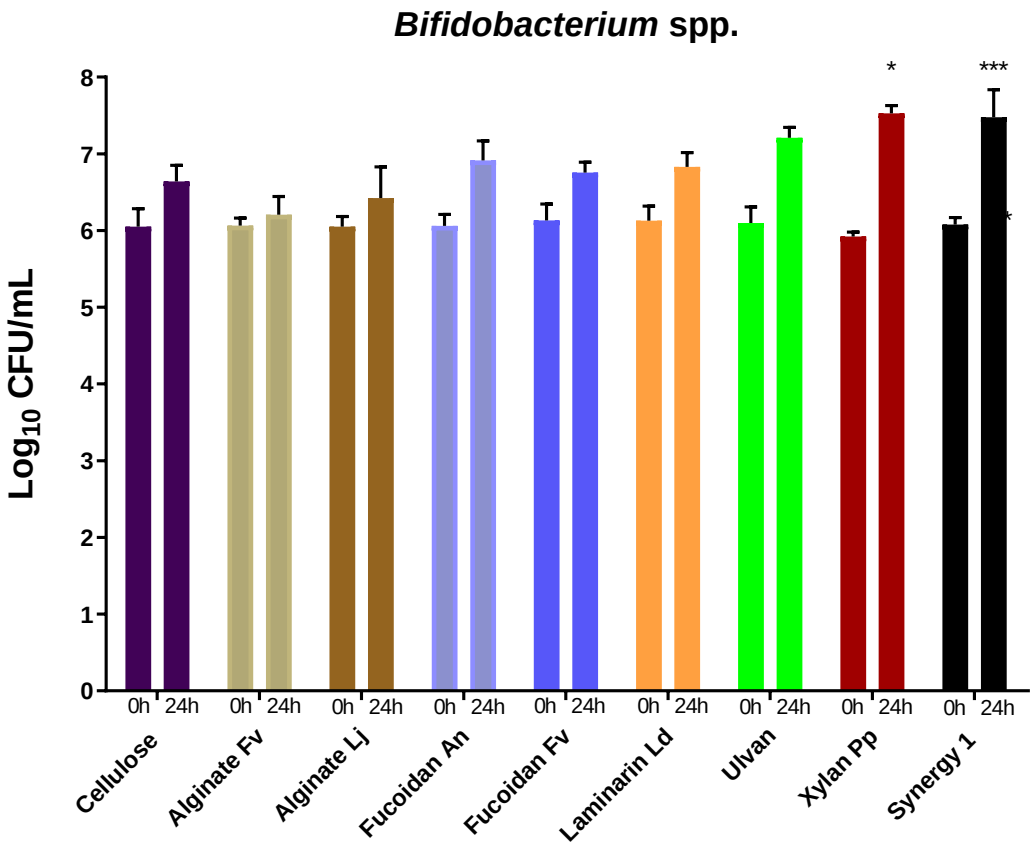

Figure 6-3. qPCR was used to quantify *Bifidobacterium* spp. from DNA extracted from fermentation vessels treated with either 1% (w/v) cellulose, or Synergy 1 substrates. Samples were taken at 0 and 24 hrs fermentation. Data plots represent the mean + standard deviation of the mean. Statistical significance was determined using a non-parametric Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). * $q \leq 0.05$, ** $q \leq 0.01$, *** $q \leq 0.005$, **** $q \leq 0.0001$.

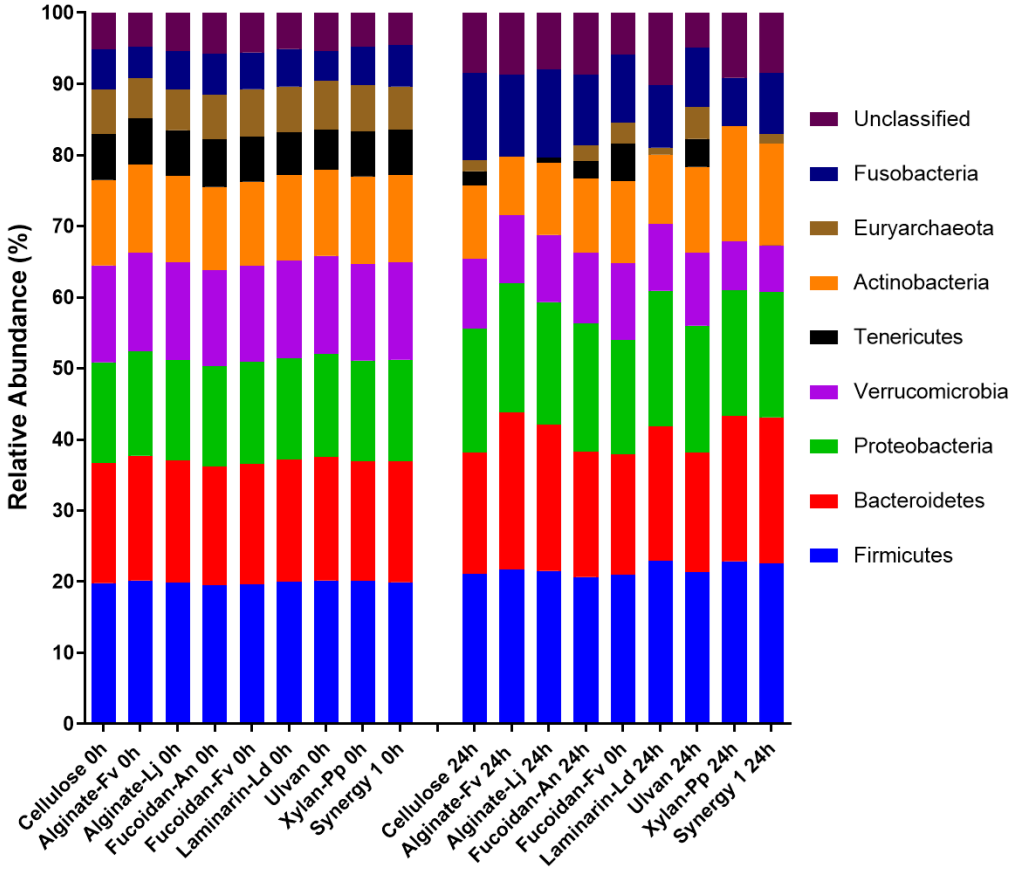

Figure 6-4. Mean relative abundances of bacterial phyla in fermentation vessels treated with either 1% (w/v) cellulose, Alginate-Fv, Alginate-Lj, Fucoidan-An, Fucoidan Fv, Laminarin-Ld, Ulvan, Xylan-Pp or Synergy 1 after 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

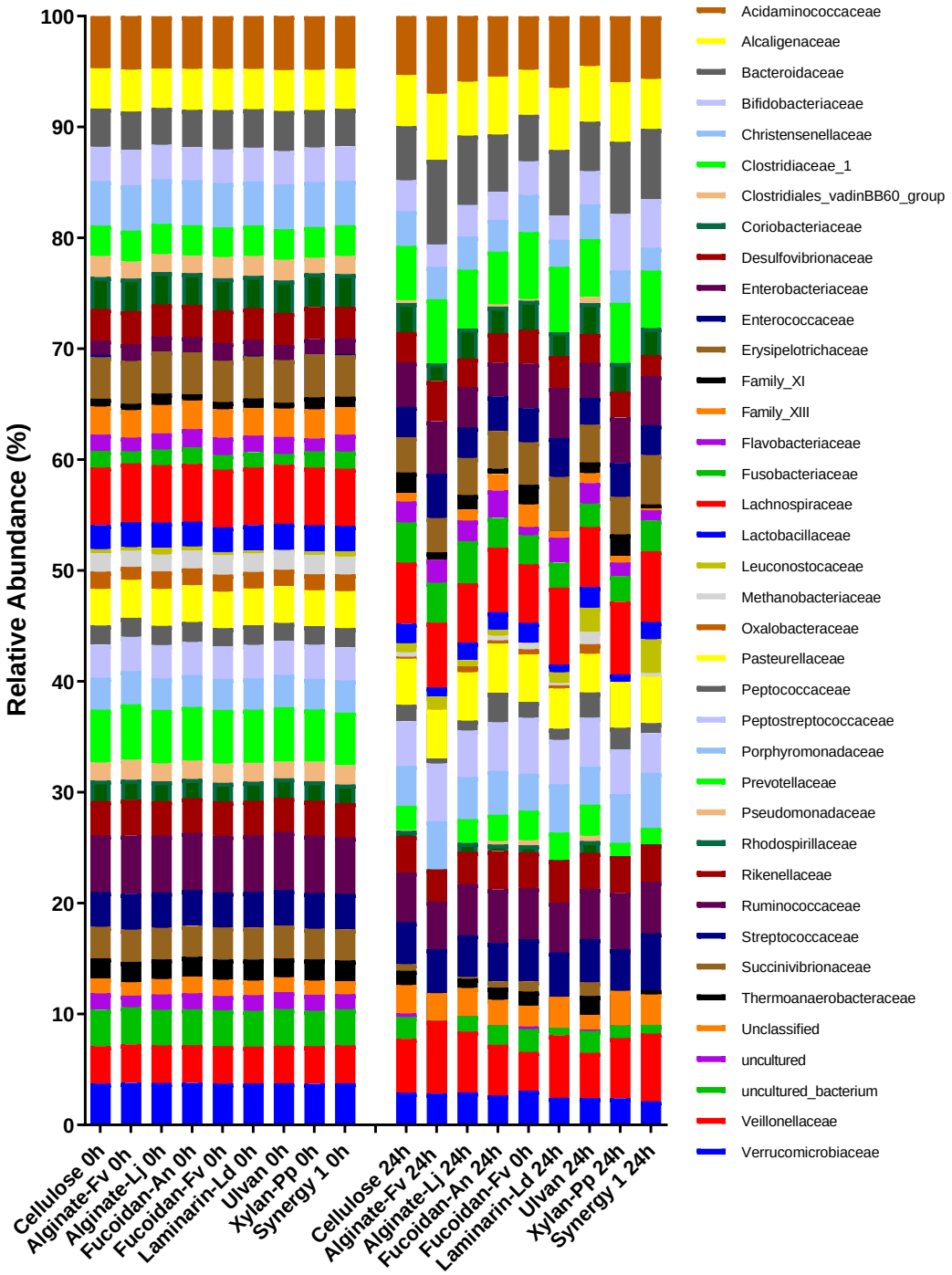

Figure 6-5. Mean relative abundances of bacterial families in fermentation vessels treated with either 1% (w/v) cellulose, Alginate-Fv, Alginate-Lj, Fucoidan-An, Fucoidan Fv, Laminarin-Ld, Ulvan, Xylan-Pp or Synergy 1 after 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

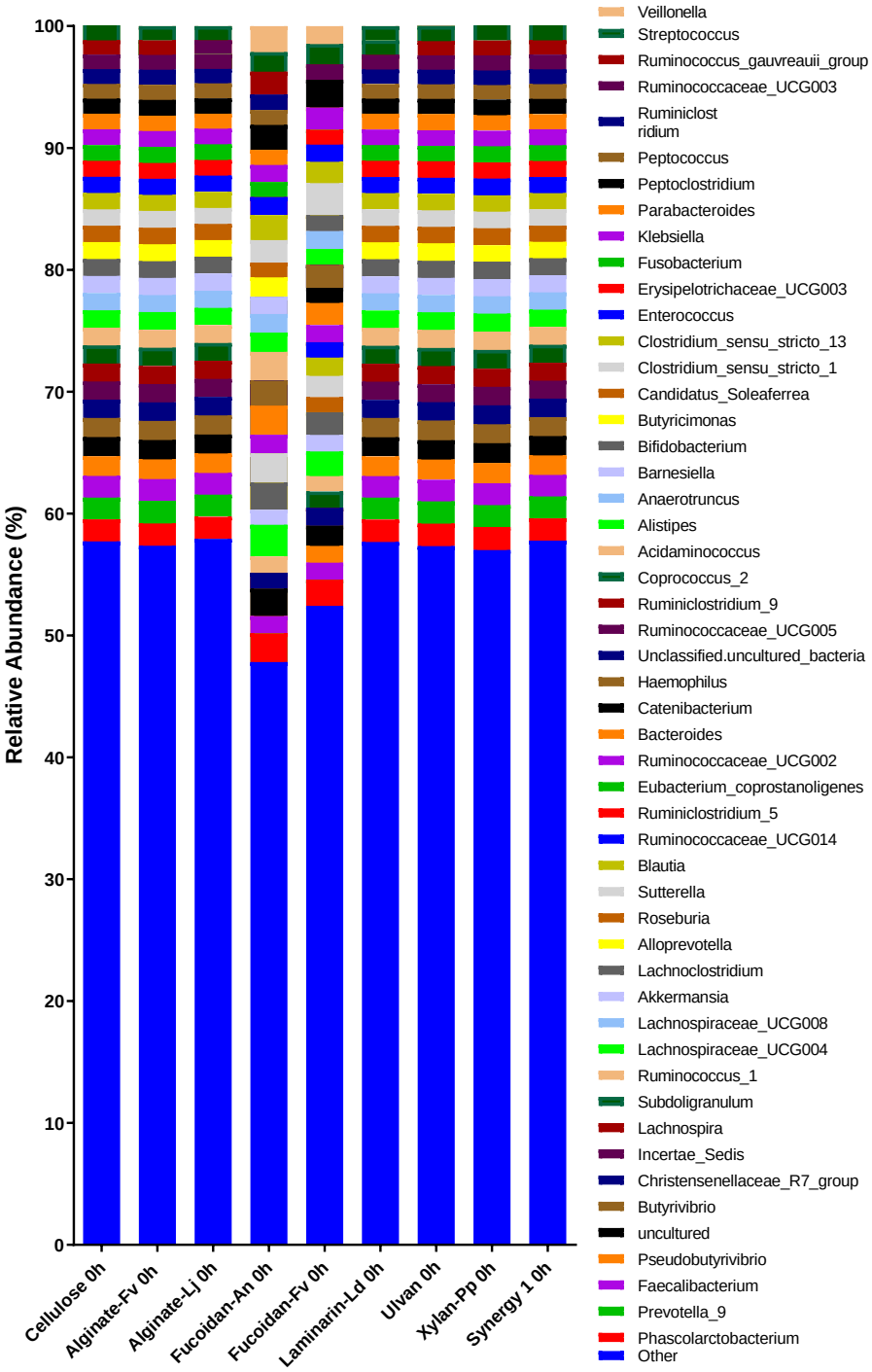

Figure 6-6. Mean relative abundances of the top 30 bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, Alginate-Fv, Alginate-Lj, Fucoidan-An, Fucoidan Fv, Laminarin-Ld, Ulvan, Xylan-Pp or Synergy 1 after 0 hrs fermentation. The percentage relative abundance of genera outside the top 30 were added together and included as "other". Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

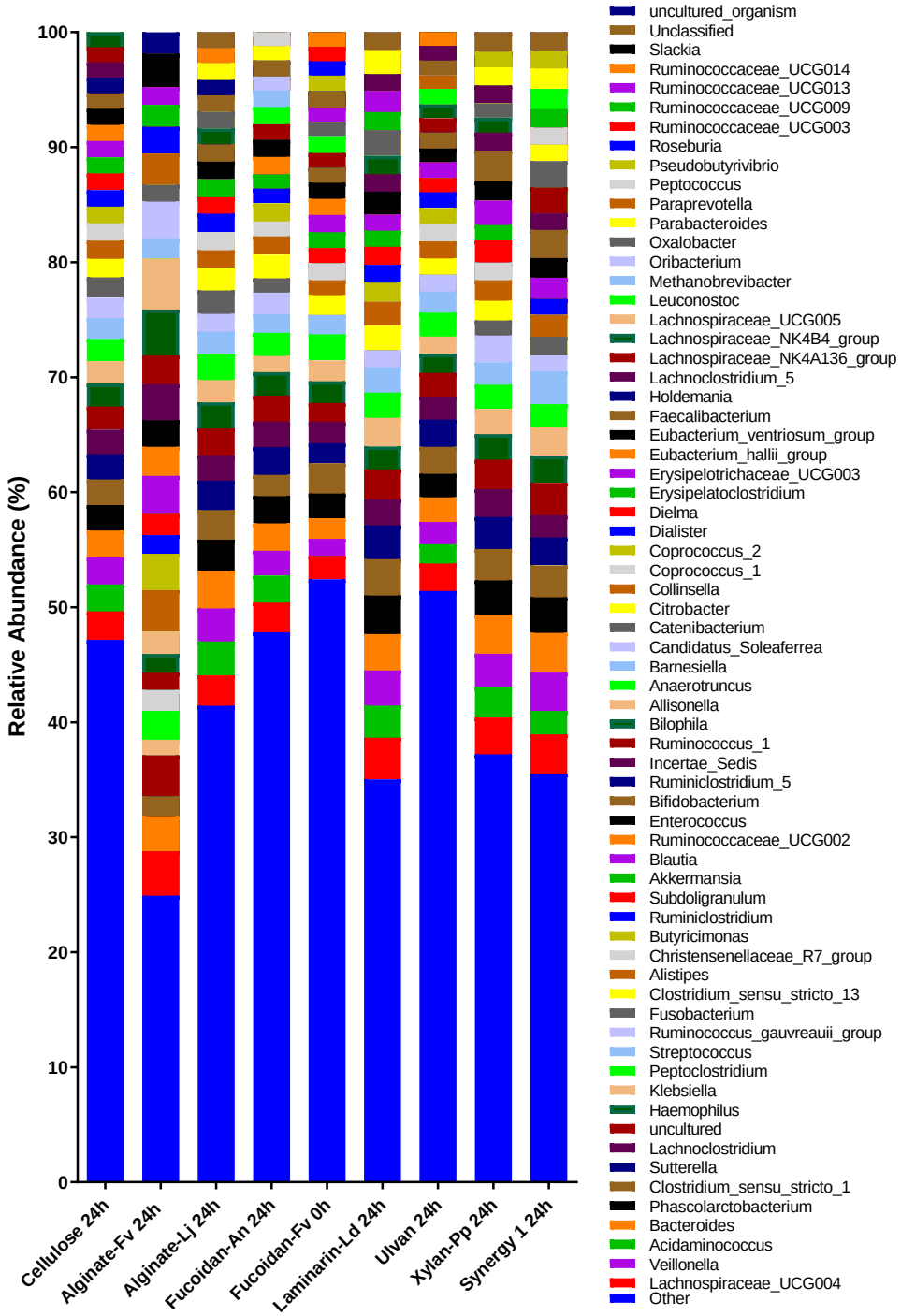

Figure 6-7. Mean relative abundances of the top 30 bacterial genera in fermentation vessels treated with either 1% (w/v) cellulose, Alginate-Fv, Alginate-Lj, Fucoidan-An, Fucoidan Fv, Laminarin-Ld, Ulvan, Xylan-Pp or Synergy 1 after 24 hrs fermentation. The percentage relative abundance of genera outside the top 30 were added together and included as "other". Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

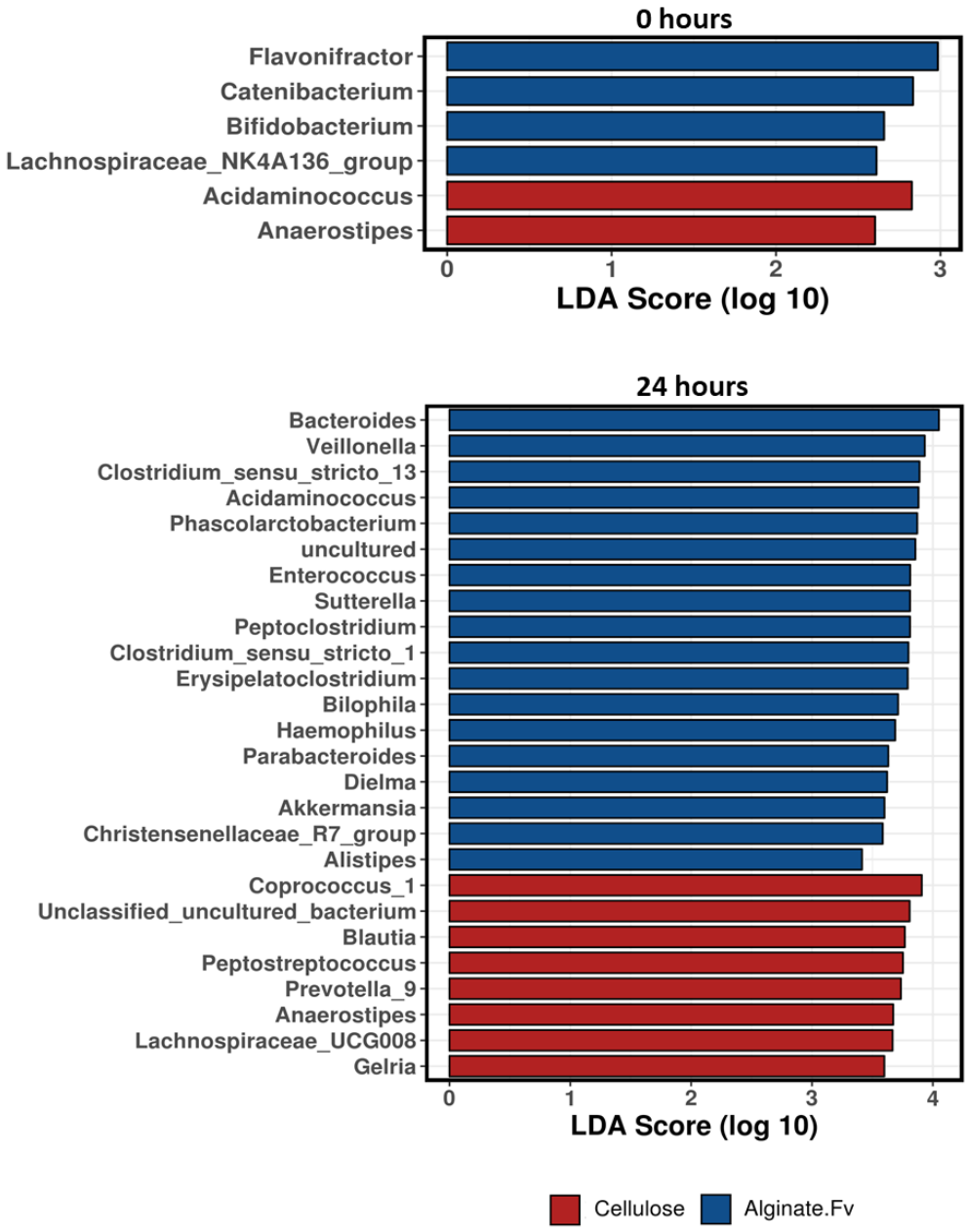

Figure 6-8. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Alginate-Fv compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

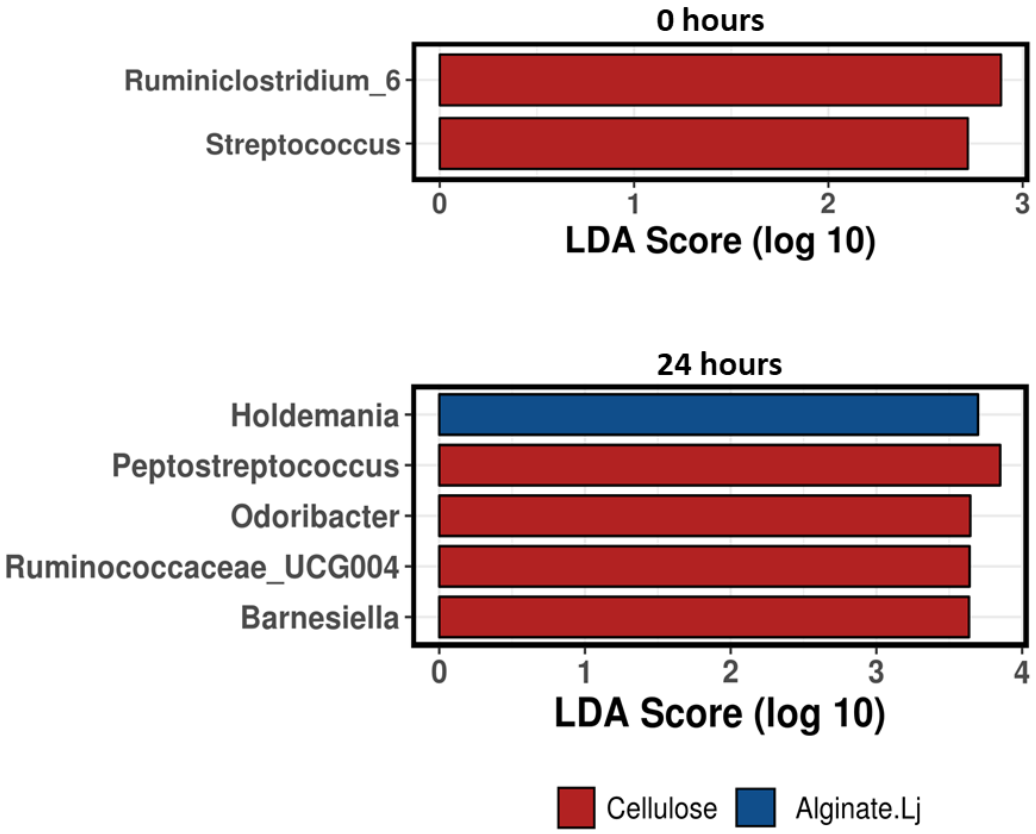

Figure 6-9. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Alginate-Lj compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

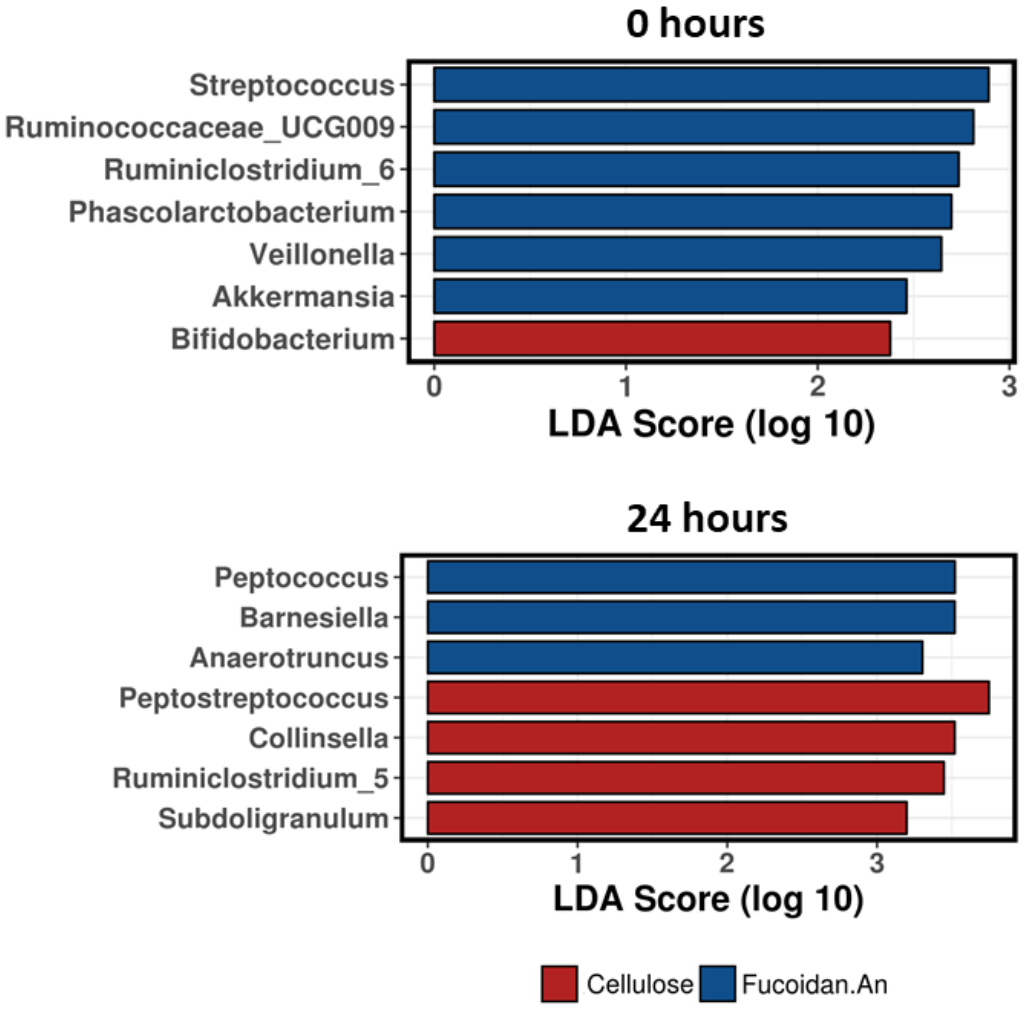

Figure 6-10. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Fucoidan-An compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

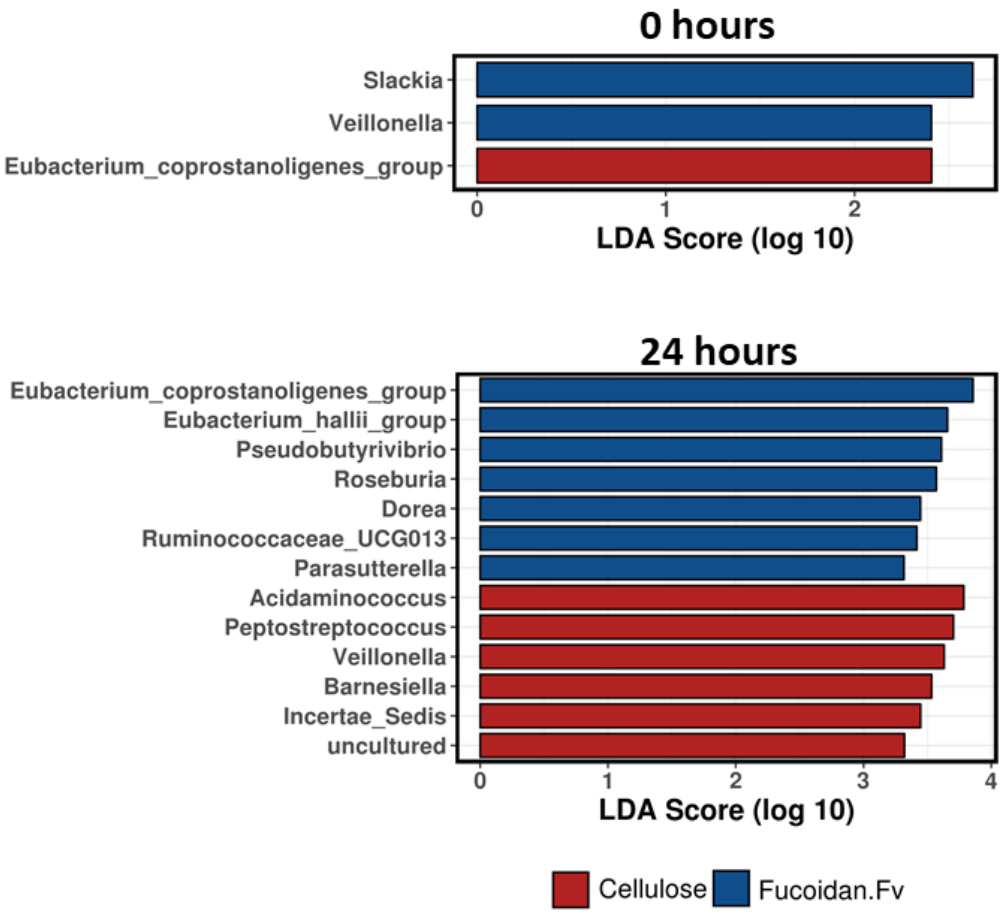

Figure 6-11. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Fucoidan-Fv compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

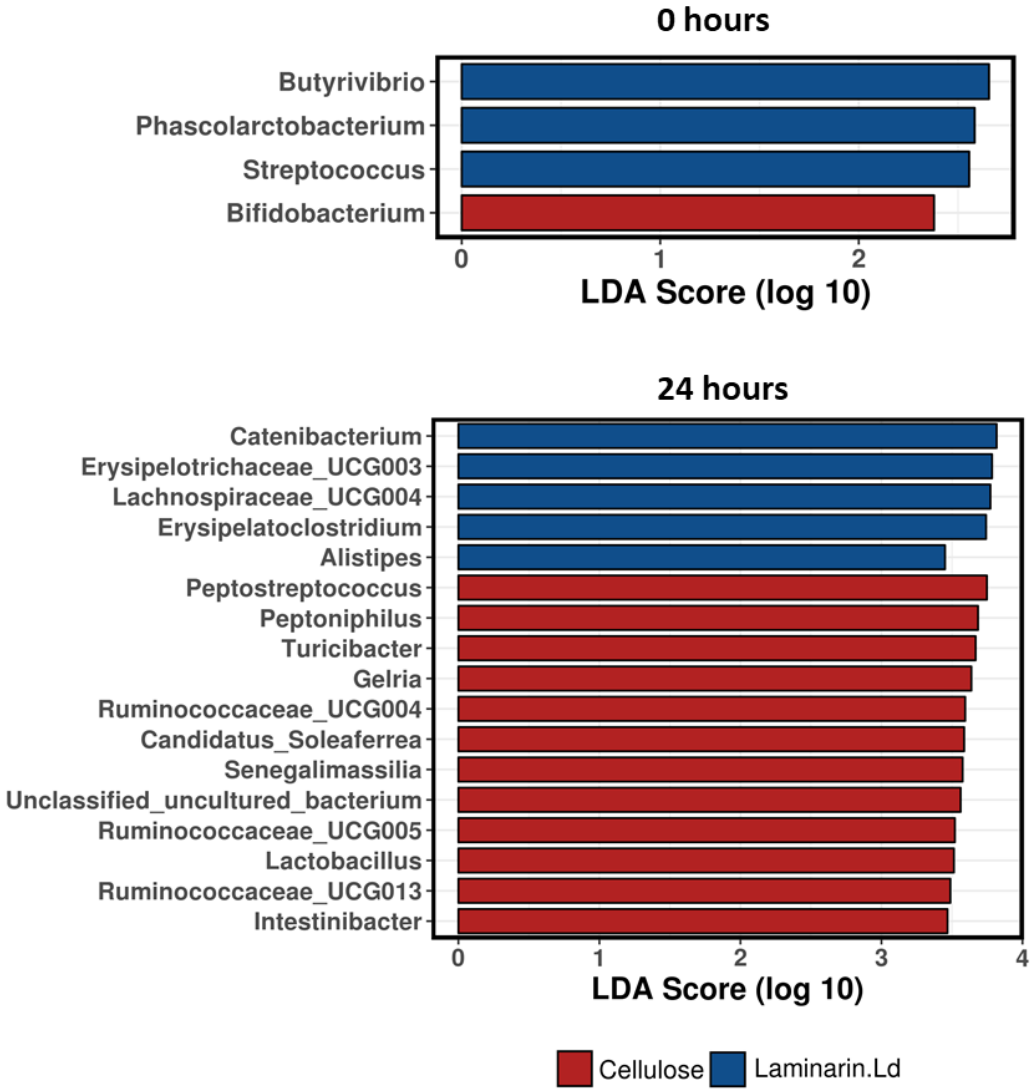

Figure 6-12. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Laminarin-Ld compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

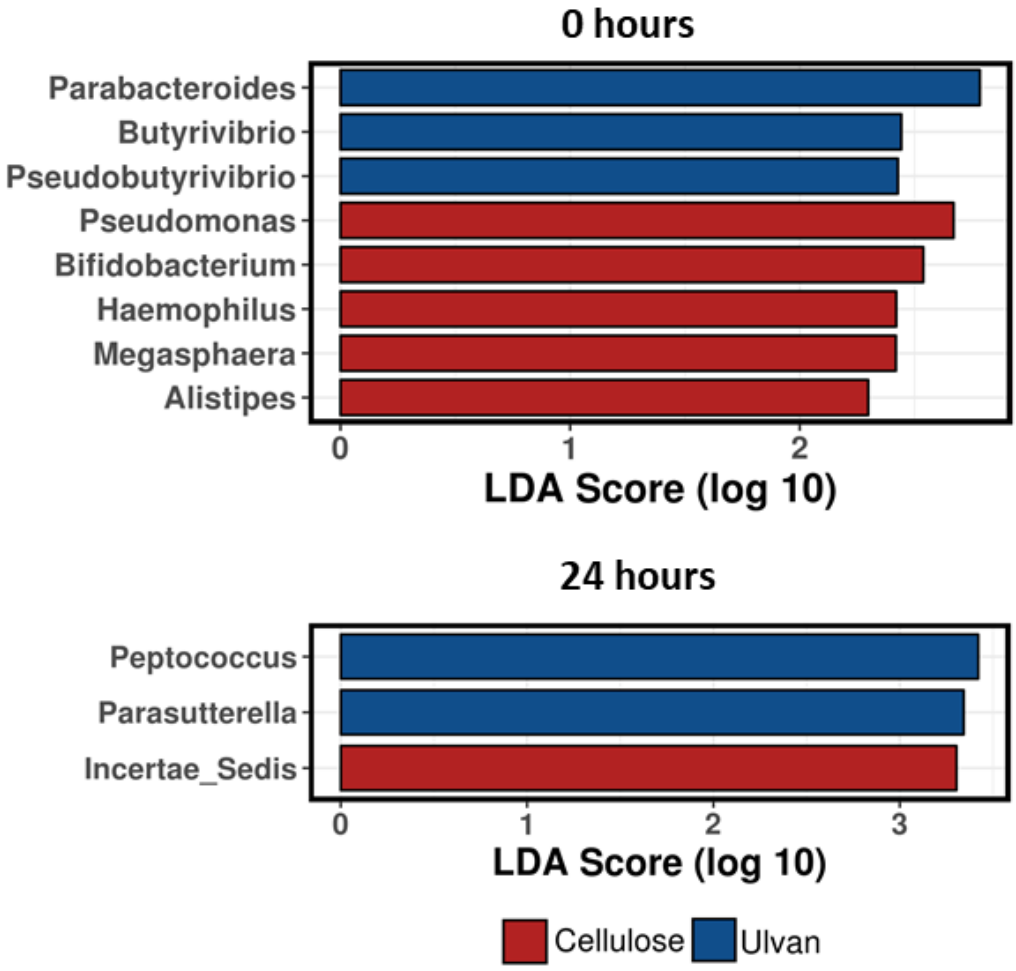

Figure 6-13. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Ulvan compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

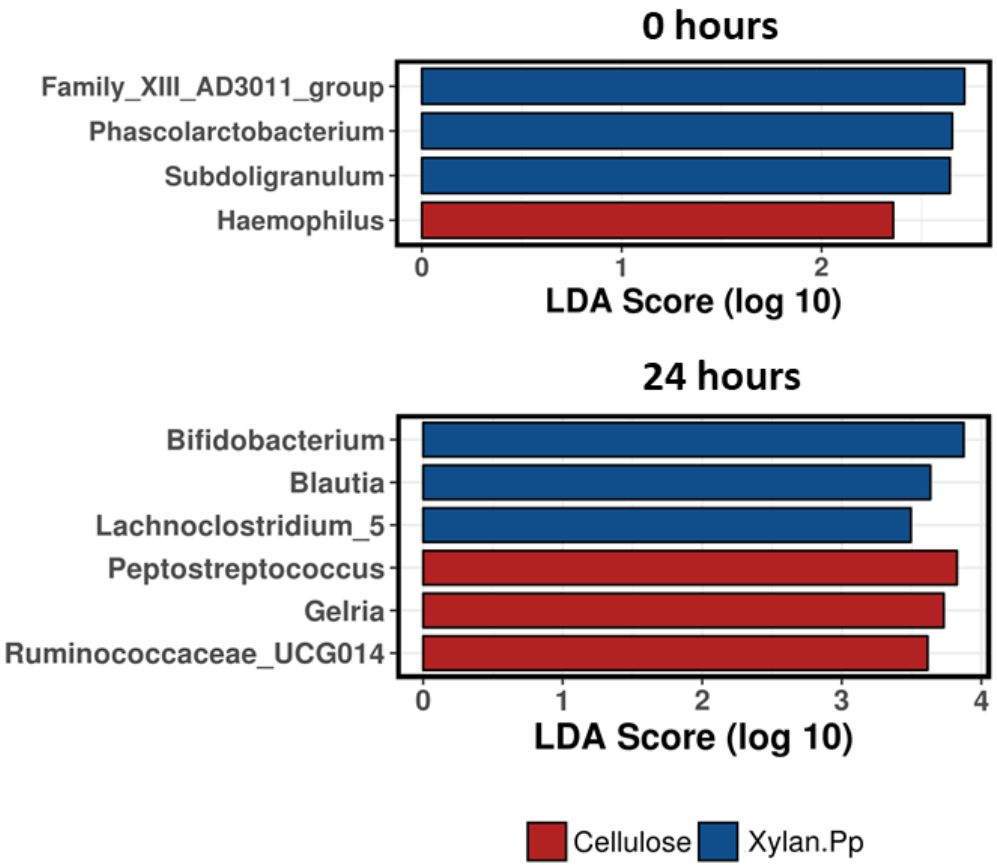

Figure 6-14. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Xylan-Pp compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

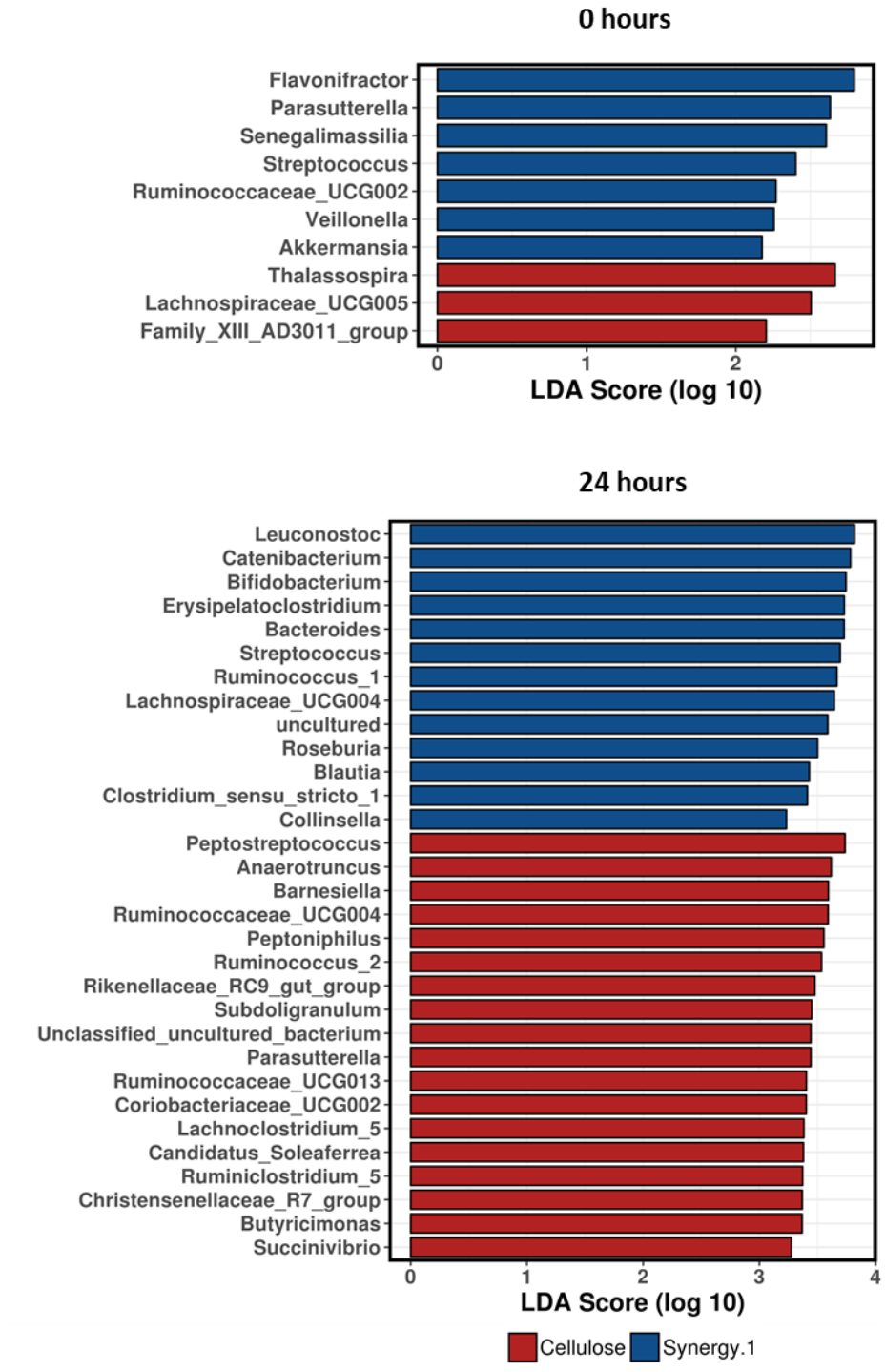

Figure 6-15. Feature selection analysis (linear discriminate analysis effect size - LEfSe analysis) was used to determine discriminative bacteria between fermentation vessels treated with 1% (w/v) Synergy 1 compared to 1% (w/v) cellulose at 0 and 24 hrs fermentation. Bacterial composition was determined using 16S rRNA sequencing on the MiSeq platform.

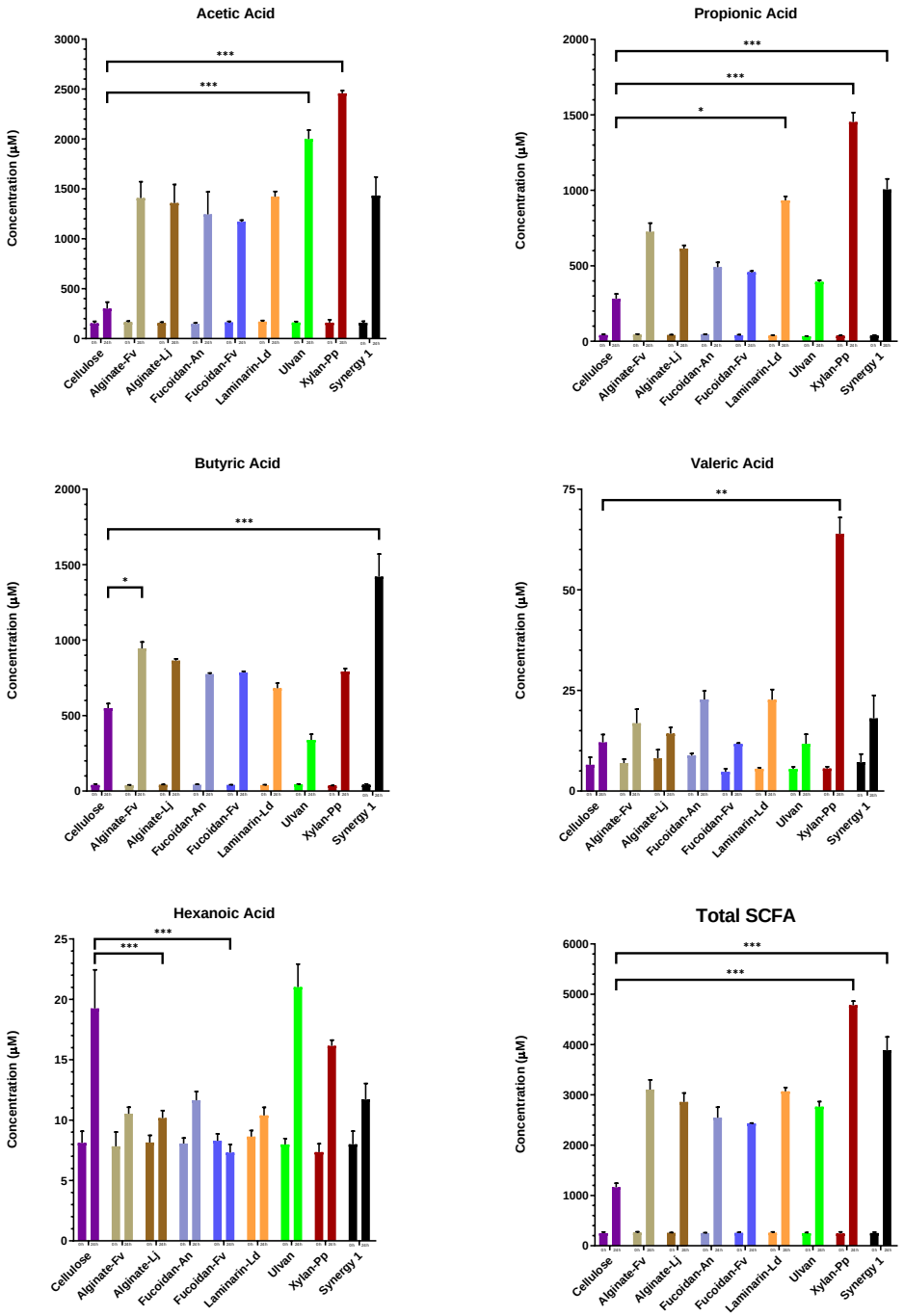

Figure 6-16. Short chain fatty acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) either 1% (w/v) cellulose, Alginate-Fv, Alginate-Lj, Fucoidan-An, Fucoidan Fv, Laminarin-Ld, Ulvan, Xylan-Pp or Synergy 1 after 0 and 24 hrs fermentation. Data plots represent the mean + standard deviation of the mean. Statistical significance was determined using a non-parametric Kruskal-Wallis test with a Bonferroni correction for multiple comparisons and a significance level of 0.05. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ when compared to cellulose at each time point.

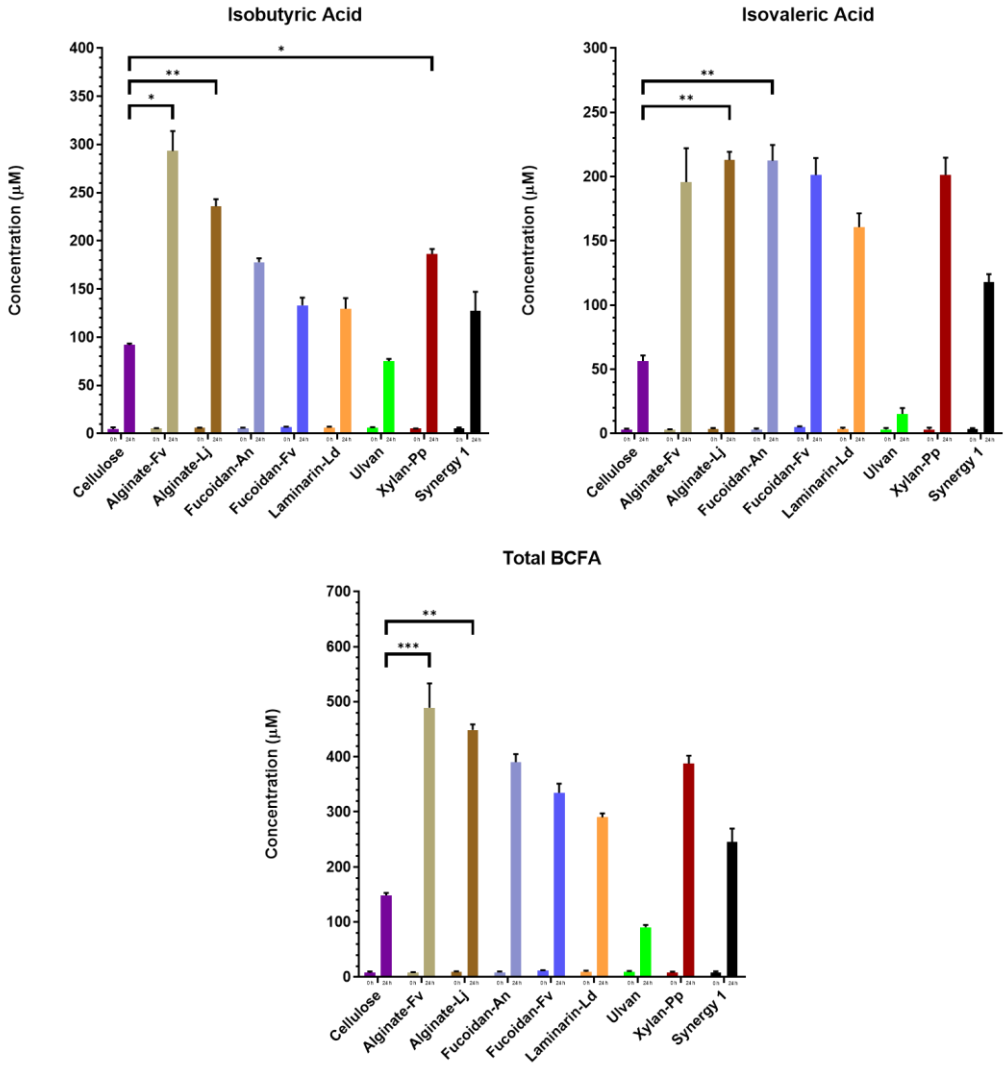

Figure 6-17. Branched chain fatty acid concentrations (µM) of fermentation vessels treated with either 1% (w/v) either 1% (w/v) cellulose, Alginate-Fv, Alginate-Lj, Fucoidan-An, Fucoidan Fv, Laminarin-Ld, Ulvan, Xylan-Pp or Synergy 1 after 0 and 24 hrs fermentation. Data plots represent the mean + standard deviation of the mean. Statistical significance was determined using a non-parametric Kruskal-Wallis test with a Bonferroni correction for multiple comparisons and a significance level of 0.05. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ when compared to cellulose at each time point.

6.9 Tables

Table 6-1. Average molecular weight of the seaweed polysaccharides used as *in vitro* fermentation substrates. Average molecular weight was determined using high performance size exclusion chromatography.

Seaweed Polysaccharide	Average Molecular Weight (kDa)
Alginate-Fv	124
Alginate-Lj	177
Fucoidan-An	850
Fucoidan-Fv	80
Laminarin-Ld	4.5
Ulvan	950
Xylan-Pp	168

Table 6-2. Statistically significant differences in the mean relative abundances of bacterial Families and Genera between fermentation vessels treated with 1% (w/v) alginate from *Fucus vesiculosus* (Alginate-Fv) compared to 1% (w/v) cellulose after 24 hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p < 0.05$. There were no statistical differences at the Phylum level of taxonomy.

	Cellulose		**Alginate-Fv**		**Effect**	***P* -Value**
	Mean (%)	**SD**	**Mean (%)**	**SD**		
Family						
Bacteroidaceae	4.877	0.792	7.658	0.494	↑	0.007186
Genus						
Bacteroides	2.335	0.53766	4.442	0.378	↑	0.023

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

Table 6-3. Statistically significant differences in the mean relative abundances of bacterial Families and Genera between fermentation vessels treated with 1% (w/v) laminarin from *Laminaria digitata* compared to 1% (w/v) cellulose after 24 hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p < 0.05$. There were no statistical differences at the Phylum level of taxonomy.

	Cellulose		**Laminarin-Ld**		**Effect**	***P*-Value**
	Mean (%)	**SD**	**Mean (%)**	**SD**		
Family						
Thermoanaerobacteraceae	1.298	0.643	< 0.001	< 0.001	↓	0.03824
Lachnospiraceae	5.503	0.416	6.932	0.407	↑	0.001007
Family XI	1.832	0.603	< 0.001	< 0.001	↓	0.014987
Erysipelotrichaceae	1.832	0.603	< 0.001	< 0.001	↓	0.04118
Genus						
Turicibacter	0.946	0.302	0.116	0.231	↓	0.028
Howardella	0.678	0.135	< 0.001	<0.001	↓	0.020
Gelria	0.669	0.266	< 0.001	<0.001	↓	0.019
Erysipelatoclostridium	0.530	0.345	1.571	0.407	↑	0.001
Catenibacterium	1.1159	0.389	2.257	0.288	↑	0.001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 6-4. Statistically significant differences in the mean relative abundances of bacterial Families between fermentation vessels treated with 1% (w/v) ulvan compared to 1% (w/v) cellulose after 24 hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p < 0.05$. There were no statistical differences at the Phylum or Genus levels of taxonomy.

	Cellulose		**Ulvan**		**Effect**	***P*-Value**
	Mean (%)	**SD**	**Mean (%)**	**SD**		
Family						
Pasteurellaceae	4.151	0.199	3.483	0.217	↓	0.035609
Fusobacteriaceae	3.639	1.045	2.125	0.740	↓	0.036499

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 6-5. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) xylan from *Palmaria palmata* (Palmaria Pp) compared to 1% (w/v) cellulose after 24 hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p < 0.05$.

	Cellulose		**Xylan-Pp**		**Effect**	***P*-Value**
	Mean (%)	**SD**	**Mean (%)**	**SD**		
Phylum						
Actinobacteria	10.300	1.530	16.180	3.230	↑	0.00173
Family						
Bifidobacteriaceae	2.797	0.472	5.105	1.044	↑	0.00017
Genus						
Bifidobacterium	1.362	0.11637	2.6858	0.671	↑	0.000199
Gelria	0.669	0.266	< 0.001	< 0.001	↓	0.042
Paraprevotella	0.583	0.222	< 0.001	< 0.001	↓	0.016

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 6-6. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) Synergy 1 compared to 1% (w/v) cellulose after 24 hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p < 0.05$.

	Cellulose		**Synergy 1**		**Effect**	***P*-Value**
	Mean (%)	**SD**	**Mean (%)**	**SD**		
Phylum						
Actinobacteria	10.300	1.530	14.290	2.040	↑	0.008046
Family						
Verrucomicrobiaceae	2.889	0.227	2.124	0.320	↓	0.001864
Streptococcaceae	3.757	0.267	5.140	0.657	↑	0.000112
Porphyromonadaceae	3.608	0.150	4.954	0.360	↑	< 0.0001
Leuconostocaceae	0.813	0.778	3.038	0.552	↑	0.000939
Lachnospiraceae	5.503	0.416	6.385	0.310	↑	0.032209
Family XI	1.832	0.603	0.344	0.445	↓	0.023453
Desulfovibrionaceae	2.713	0.393	1.864	0.181	↓	0.004723
Christensenellaceae	3.131	0.3410	2.052	0.653	↓	0.006843
Bifidobacteriaceae	2.797	0.4720	4.388	0.665	↑	0.000883

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Table 6-6 continued. Statistically significant differences in the mean relative abundances of bacterial Phyla, Families and Genera between fermentation vessels treated with 1% (w/v) Synergy 1 compared to 1% (w/v) cellulose after 24 hrs fermentation. Statistical significance was determined using a one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons and a significance level of $p < 0.05$.

	Cellulose		**Synergy 1**		**Effect**	***P*-Value**
	Mean (%)	**SD**	**Mean (%)**	**SD**		
Genus						
Erysipelatoclostridium	0.530	0.345	1.591	0.318	↑	< 0.0001
Paraprevotella	0.583	0.222	< 0.001	< 0.001	↓	0.001
Ruminococcus 1	1.314	0.097	2.251	0.528	↑	0.039
Streptococcus	1.818	0.322	2.855	0.846	↑	0.024
Leuconostoc	0.482	0.459	1.778	0.613	↑	0.009
Howardella	0.678	0.135	0.130	0.226	↓	0.031
Desulfovibrio	0.659	0.114	0.086	0.228	↓	0.025
Catenibacterium	1.115	0.3888	2.284	0.300	↑	< 0.0001
Bifidobacterium	1.362	0.116	2.442	0.568	↑	< 0.0001

↑ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly higher in seaweed treated vessels when compared to cellulose

↓ The mean relative abundance of a given Phylum, Family, or Genus was statistically significantly lower in seaweed treated vessels when compared to cellulose

Chapter 7

General Discussion

7 General Discussion

The aims of this thesis were to evaluate the fermentability of seaweeds and their extracted polysaccharides, using *in vitro* batch culture fermentation models, and to assess whether this fermentation could modulate gut microbiota composition and metabolic activity, conducive to a prebiotic effect. Batch culture fermentation substrates included whole seaweeds (a food matrix), polysaccharide-rich extracts (a mixture of dietary fibre components), and purified seaweed polysaccharides (individual dietary fibre components).

Whole seaweed powders of the red seaweed *Palmaria palmata* and the brown seaweeds *Fucus vesiculosus* and *Laminaria digitata* were chosen because this is the form of the seaweed most typically consumed in food products. However, owing to potentially high levels of trace elements such as iodine and arsenic in whole seaweeds, crude polysaccharide-rich extracts were generated from the whole seaweed powders using a low-cost chemical extraction method. Commercially available and purified forms of alginate, fucoidan, and laminarin (brown seaweed polysaccharides), ulvan (green seaweed polysaccharide) and xylan (red seaweed polysaccharide) were chosen to understand the effect of individual seaweed polysaccharides on gut microbiota composition and metabolic activity, rather than a crude mixture. Moreover, it was envisaged that data obtained in **Chapter 6** could provide scope for future investigations akin to terrestrial plant derived complex polysaccharides (e.g. inulin) and oligosaccharides (e.g. fructooligosaccharides, galctooligosaccharides, and xylooligosaccharides), given that methods to extract and purify individual polysaccharides from seaweeds are currently expensive (Jönsson et al., 2020).

Dietary intake of seaweeds has been associated with the reduced risk of non-communicable diseases, owing to the bioactivity of biomolecules present in the food matrix, such as complex polysaccharides, proteins, polyphenols, and polyunsaturated fatty acids (Brown et al., 2014; Peñalver et al., 2020). This has generated significant interest in the use of seaweeds as functional food ingredients (Peñalver et al., 2020), where functional foods are defined as a food which "is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Diplock et al., 1999).

Chapter 2 of this thesis evaluated the nutritional composition of seaweeds and explored the current evidence from studies which have investigated how dietary intake of seaweeds and seaweed phytochemicals may impact host physiology to benefit health. It is well established that seaweeds can provide a meaningful contribution to nutritional intakes of micronutrients including vitamins and minerals, and certain macronutrients including dietary fibre and protein, albeit this is highly variable between seaweed sources (Bouga and Combet, 2015; Brown et al., 2014; MacArtain et al., 2007). This literature review summarised the evidence relating to the health benefits of seaweed beyond the provision of essential nutrients and demonstrated where these benefits are supported by pre-clinical studies, particularly in the context of individual seaweed components benefiting cardiometabolic health. For instance, there is evidence to suggest that fucoidan and alginate polysaccharides have anti-obesogenic properties; polyphenols may

positively impact glucose homeostasis and lipid digestion/metabolism; and polyunsaturated fatty acids may exert anti-inflammatory and anti-obesity effects.

This literature review also concluded that the number of human intervention studies with appropriate health related endpoints is scarce and moreover, that the variable and potentially high concentrations of salt, iodine, and arsenic contents in seaweeds can limit the portion size an individual can consume. Thus, the extraction and isolation of bioactive components from seaweeds represents an opportunity to create value-added functional food ingredients, while mitigating excessive trace element intakes. Hence the reason for mineral and trace element analysis of the seaweed powders and polysaccharide-rich extracts generated for the experiments contained within this thesis.

The evidence presented in **Chapter 3** highlights the range of polysaccharides present in seaweeds, such as alginate, fucoidan, and laminarin in brown seaweeds; agar, carrageenan, porphyran, and xylan in red seaweeds; and ulvan in green seaweeds. The findings indicated that the human gut microbiome does express some carbohydrate active enzymes (CAZymes) which can break down seaweed polysaccharides to facilitate fermentation. This literature review also indicated that the most promising prebiotic candidates were alginate, laminarin, and red seaweed galactans, owing to more evidence showing that their polysaccharides and oligosaccharides elicited favourable changes to microbiota composition and metabolic output. Nevertheless, there were also knowledge gaps identified in this literature review, including the lack of human studies that have investigated dietary interventions with seaweed polysaccharides as putative functional food ingredients.

Another outcome of this literature review was that experiments are needed to understand the structure-function relationships of the fermentation of seaweed polysaccharides, owing to variability in seaweed polysaccharide structure and differential effects on gut bacterial composition and metabolic output (typically SCFA concentration). Therefore, the seaweed powders and polysaccharide extracts generated for this thesis were obtained from seaweeds at the same geographical location, time of year, and were extracted using the same method. Furthermore, attempts were made to characterise the molecular weight and polysaccharide contents of the crude polysaccharide-rich extracts, to explain which components were fermented, if any.

The data obtained in **Chapter 4** showed that the brown seaweeds *Fucus vesiculosus and Laminaria digitata* contained fermentable components, owing to significantly higher concentrations of short chain fatty acids when compared to the poorly fermented negative control cellulose. This suggests that there were bacterial communities present in the *ex vivo* faecal microbiota that expressed CAZYmes that could break down the constituent polysaccharides present e.g. alginate, fucoidan, and laminarin. This is supported, in part, by the data obtained in **Chapter 6**, which showed that the fermentation of laminarin and alginate polysaccharides yielded significantly higher concentrations of propionic acid and butyric acid, respectively.

Although fucoidan showed limited fermentability (**Chapter 6**), there may be scope to investigate higher doses of substrate and the structure-function effects on the fermentability of fucoidan because there is a dearth of studies which have investigated the effects of fucoidan on the gut microbiota (**Chapter 3**). This is surprising given the FDA GRAS status and EU novel food status of fucoidan,

alongside the purported anti-obesity effects of dietary intakes of fucoidan (**Chapter 2**) and the role of diet-microbe-host interactions on metabolic disease (European Commission, 2017; FDA, 2016; Hernandez-Corona et al., 2014; Kim et al., 2014; Lim et al., 2017; O'Grady et al., 2019).

The stimulation of glycan degrading bacterial families such as *Bacteroidaceae, Ruminococcaceae* and *Lachnospiraceae* in vessels treated with whole brown seaweed powders and polysaccharide extracts when compared to cellulose supports previous pre-clinical evidence (Choa An et al., 2013; Shang et al., 2016; Strain et al., 2019). This is also supported by the data obtained in **Chapter 6** which demonstrated that purified alginate and laminarin substrates stimulated populations of *Bacteroides* and *Lachnospiraceae*, respectively. This may be explained by the expression of alginate lyase and laminarinase CAZymes present in the genome of *Bacteoides* spp. (Li et al., 2017; Maruyama et al., 2015; Mathieu et al., 2018, 2016; Salyers et al., 1977; Thomas et al., 2012).

Human gut *Bacteroides* function as primary fermenters to facilitate cross-feeding for syntrophic bacteria such as *Phascolarctobacterium* spp., which convert succinate to acetate and propionate (Fischbach and Sonnenburg, 2011; Wu et al., 2017). Given that *Phascolarctobacterium* were stimulated by both the FVE and LDE substrates in **Chapter 4** and were a discriminative genus of Alginate-Fv fermentation in **Chapter 6,** it can be postulated that human gut *Bacteroides* were able to degrade alginate components present in the seaweed and seaweed extract, to cross feed for other stimulated genera such as the butyric acid producers *Pseudobutyrivibrio*, *Butyricimonas* and *Coprococcus*, which were also increased by the *Laminaria digitata* polysaccharide extract. The concept of cross-feeding was

recently suggested for the traditional markers of a prebiotic, since bifidobacteria and lactobacilli do not produce butyrate directly, rather acetate and lactate (Scott et al., 2013). Thus, the recycling of metabolites facilitates cross-feeding by acetate/lactate-consuming butyrate-producing Firmicutes such as *Roseburia spp*., *Butyricicoccus spp*., *Anaerostipes spp*., and *Faecalibacterium prausnitzii* (Allen-Vercoe et al., 2012; Eeckhaut et al., 2013; Kant et al., 2015; Miquel et al., 2013).

Another knowledge gap identified in **Chapter 3** showed the paucity of studies regarding the fermentability of xylan obtained from the red seaweed *Palmaria palmata*. The data obtained in **Chapter 5** indicated that the whole seaweed powder contained fermentable components owing to a significantly higher concentration of SCFA when compared to cellulose, whereas the polysaccharide extract did not. The noted differences can most likely to be explained by the combination of components in the whole seaweed matrix provided a range of substrates for bacteria when compared to polysaccharide extract alone, an observation which was also previously reported for oat bran compared to its isolated beta glucans and polyphenols (Kristek et al., 2019). Despite this, fermentation of the purified Xylan-Pp substrate investigated in **Chapter 6** resulted in significantly higher concentrations of acetic, propionic, valeric and total short chain fatty acids when compared to cellulose. The difference in effect on SCFA observed between the purified xylan and the *Palmaria palmata* polysaccharide-rich extract may be owing to fact that the purified compound was 100% xylan, whereas the extract was a mixture of polysaccharide such as cellulose and xylan. This is a limitation of using a crude extract that lacked full chemical characterisation, although it is envisaged that the low molecular weight carbohydrate, floridoside,

was removed from the *Palmaria palmata* polysaccharide-rich extract during the extract production and *in vitro* simulated digestion.

Chapter 5 also showed that both the seaweed powder and polysaccharide-rich extract of *Palmaria palmata* stimulated the butyrate-producing genus, *Butyricimonas*, which also occurred with the brown seaweed substrates in **Chapter 4**. Furthermore, the *Palmaria palmata* seaweed powder and polysaccharide-rich extract elicited a bifidogenic effect, and in **Chapter 6,** the purified Xylan-Pp substrate also elicited a bifidogenic effect. It may therefore be postulated that human gut *Bifidobacterium* spp. expressed β-(1→3)-xylosidase and α-L-arabinofuranosidase CAZymes to break down the xylan components in each test substrate (Kobayashi et al., 2020).

The evidence of a bifidogenic effect of xylan from *Palmaria palmata* is perhaps the most novel and impactful finding from this thesis. Given that the stimulation of bifidobacteria is a widely accepted indicator of a potential prebiotic, the noted changes induced by *Palmaria palmata* extracts and commercially available xylan supports the need for future work to scale up the production of purified xylans and xylooligosaccharides from *Palmaria palmata* to investigate their effects on the gut microbiota and host health *in vivo*.

Chapter 6 also showed that the green seaweed polysaccharide, ulvan resulted in significantly higher concentrations of acetic acid which indicates fermentability. This was also shown in another *in vitro* fermentation study (Seong et al., 2019). However, no significant changes in bacterial composition were noted in the present study, which is in contrast to the promotion of *Bifidobacterium*, *Lactobacillus* and *Akkermansia muciniphila* populations elsewhere (Seong et al.,

2019; Shang et al., 2018). Given the paucity of studies which have investigated the fermentability of ulvan (**Chapter 3**), further investigation is needed to understand whether this green seaweed polysaccharide is fermented by human gut bacterial populations given the purported anti-inflammatory effects of dietary intakes of ulvan (Ren et al., 2017).

The use of a single stage closed system batch culture fermentation system in this study was useful for screening purposes, owing to its speed and simplicity to use. However, the lack of mucus and physiological relevance to the proximal and transverse colon present limitations of this model because they do not represent the complex gut microbiota ecosystem *in vivo* (Pham and Mohajeri, 2018).

Cellulose was selected as the negative control in the experimental chapters of this thesis because it is poorly fermented by the human gut microbiota and is not a prebiotic (Gibson et al., 2017). While several studies have also used cellulose as a negative control (Charoensiddhi et al., 2017, 2016; de Godoy et al., 2015; Kaewmanee et al., 2019), it is recognised that a low level of fermentation does occur. Therefore, the use of a no substrate control may be a more appropriate negative control (Guergoletto et al., 2016). For the data obtained in this thesis, statistical analysis compared to the 0 hrs time point for each given substrate would be an alternative approach. In future, the use of cellulose as a non-fermentable fibre control, alongside a no substrate control, could be optimal (Han et al., 2020; Tsitko et al., 2019).

By 2026, the global commercial seaweed and global prebiotics markets are estimated to be worth $85 billion and $8.5 billion, respectively (Ahuja and

Mamtani, 2019; Pulidindi and Prakash, 2020). The seaweed industry will be pivotal for the development of sustainable global food resources (Kraan, 2013; Mac Monagail et al., 2017), while the rapidly developing area of prebiotics has implications on human nutrition and health. This has focused on functional food ingredients which improve gastrointestinal health, glycaemic control, bone health, and improve weight management (Gibson et al., 2017). Therefore, the data obtained, and the discussion points raised throughout this thesis, have wider implications within two growing commercial markets that require innovation and investment.

7.1 Conclusion

Extractable seaweed polysaccharide components have been suggested as potential value-added ingredients for the blue bioeconomy and functional food industry, owing to purported bioactivities of non-digestible carbohydrates and the opportunity to mitigate excessive iodine intake associated with consuming seaweeds.

The data obtained from this thesis provides evidence that brown seaweeds and red seaweeds contain microbiota accessible components that are fermented by human gut microbiota populations to elicit changes to bacterial composition and metabolic output. It is postulated that the changes observed were due to the presence of alginate and laminarin (brown seaweeds) and xylan (red seaweed, *Palmaria palmata*) polysaccharides.

Future opportunities should develop commercially viable and low-cost production methods to obtain purified seaweed polysaccharides and oligosaccharides, akin to marketed terrestrial plant fibre ingredients. This will facilitate *in vivo* animal studies

and dietary human intervention studies to understand the impact of seaweed poly- and oligosaccharides on gut microbiota composition and metabolic activity, and to understand whether such modulation can confer a benefit to host health.

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Appendices

8 Appendix 1 – Experimental materials and methods

8.1 Chemicals and reagents

Chemicals were purchased from Sigma Aldrich (USA) unless otherwise stated. Reagents used during High Performance Size-Exclusion Chromatography (HPSEC) and Gas Chromatography Mass Spectrometry (GC-MS) were HPLC grade. Reagents used for DNA extraction, quantitative PCR (qPCR), and 16S rRNA next generation sequencing were molecular biology grade.

8.2 Seaweed fermentation substrates

8.2.1 Seaweed harvest

Fucus vesiculosus, Laminaria digitata, and *Palmaria palmata* were harvested from Spiddal, Co. Galway, Ireland (53° 14' 48" North, 9° 18' 10" West) in September 2014. Only the leaf was used, and the stipe was removed from the holdfast during harvesting. The seaweed was immediately washed in seawater to remove contaminants (e.g. epiphytes, molluscs and other seaweeds). Washed seaweed was freeze-dried, ground into a fine powder using an electronic blender, and stored at -20 °C until required.

8.2.2 Polysaccharide-rich extract production

Fucus vesiculosus, Laminaria digitata, and *Palmaria palmata* powders were shaken independently in dH_2O (1:20, w/v) for two mins to reduce the initial salt content of the seaweed and then filtered through muslin cloth to remove the water. 30g of washed whole seaweeds were immediately freeze-dried and stored for *in vitro* digestion and batch culture fermentation experiments. Seaweed powders were then shaken independently in 0.1 M HCl solution (1:10, w/v) at 170 rpm, 60°C for three hrs in the Labnet 311DS shaking incubator (Labnet International, New Jersey,

USA). The mixture was filtered through muslin cloth and filtrates were centrifuged at 3696 RCF for 10 mins to separate additional residue. The extraction protocol was then repeated on the residue, giving a total extraction time of six hrs. Filtrate supernatant was immediately collected and neutralised using 2 M NaOH to reduce the opportunity for depolymerisation. The neutralised aqueous extract was then desalinated using the MemBrain P EDR-Z lab-scale electrodialysis unit (MemBrain, Czech Republic). Conductivity, as an indicator of salinity, was recorded using the HI-8733N Conductivity Meter (Hannah Instruments, USA). The reduction of extract salinity was recorded as follows: *Fucus vesiculosus* from 12.22 mS/cm to 1.43 mS/cm after seven hrs; *Laminaria digitata* from 18.32 mS/cm to 0.76 mS/cm after six hrs; and *Palmaria palmata* from 17.91 mS/cm to 0.37 mS/cm after 4.5 hrs.

The neutralised, desalinated, aqueous solution was freeze-dried using the FreeZone 12 plus freeze-dry system with stoppering tray dryer (Labconco, USA). The resultant powder was precipitated in 96% ethanol (1:5, w/v) and centrifuged at 3696 RCF for 10 mins. The supernatant was carefully decanted and ethanol insoluble precipitates (pellets) were dried in a fume hood overnight to remove residual ethanol. Dried pellets were then lyophilised to obtain a polysaccharide-rich extract powder of *Fucus vesiculosus* (FVE)*, Laminaria digitata* (LDE)*,* and *Palmaria palmata* (PPE)*.*

8.2.3 Individual seaweed polysaccharides

Fucoidan from *Fucus vesiculosus* (F5631) and Laminarin from *Laminaria digitata* (L9634) were purchased from Sigma Aldrich. Alginate from *Fucus vesiculosus* (ALG101), Alginate from *Laminaria japonica* (ALG100), Fucoidan from *Ascophyllum nodosum* (FUC400), Ulvan from *Ulva* spp. (ULV100), and Xylan from *Palmaria*

palmata (XYL100) were purchased from Elicityl (France). These substrates were stored sealed at room temperature until required for *in vitro* digestion and *in vitro* fermentation experiments.

8.3 Seaweed extract characterisation

Average molecular weight of each polysaccharide extract was determined using modified methodology from Gómez-Ordónez *et al.* (Gomez-Ordonez et al., 2012) using high performance size exclusion chromatography (HPSEC) at the Institute of Technology, Sligo. 50mM ammonium formate was made using Millipore Milli-Q Ultrapure water, vacuum-filtered through 0.45µm filter and used for the preparation of all solutions and the mobile phase. A pullulan standard set with molecular masses of 708, 344, 194, 107, 47.1, 21.7, 10.0, 6.2, 1.32 and 0.34 kDa was used to perform system calibration and molecular weight estimation. Standards were separately injected at 0.5 mg/mL in 50mM ammonium formate buffer in triplicate. A standard calibration curve for the logarithm of the molecular weight versus the HPSEC retention time was obtained for each series of standards (retention volumes are directly proportional to the logarithm of their relative molecular mass). Instrument and chromatographic conditions: The HPLC system was equipped with the Dionex Ultimate 3000 pump system and autosampler, Varian 385LC Evaporative Light Scattering Detector and Chromeleon control software. Separation was performed on a PL aquagel-oh mixed-H 8µm SEC analytical column (7.5 x 300 mm i.d) with isocratic elution at 50°C and a flow rate of 0.6 mL/min. 10µL of standard or sample (0.5 mg/mL) was injected into the HPLC with a run time of 31 mins.

Total carbohydrate content of each extract was quantified using a modified phenol-sulfuric acid colorimetric assay (DuBois et al., 1956). Extracts were dissolved 1:20 (v/v) in dH_2O and 25µL liquefied phenol was added, followed by 2.5mL concentrated sulphuric acid and incubated at room temperature for 20 mins. Absorbance was measured at 490nm (hexoses) and 480nm (pentoses) and concentration was interpreted from a standard curve of glucose and xylose ranging from 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1mg/mL.

Total sulphate content of each seaweed extract was measured using the Azure A method described by Torode *et al.* (Torode et al., 2015) with modifications. 75µL of 2.5M concentrated H_2SO_4 was added to 75µL of 2mg/mL seaweed extract followed by 1350µL of Azure A solution. Solutions were vortexed briefly and incubated at room temperature for 10 mins, where a blue to purple colour change occurred following ionic complex formation between anionic sulphated polysaccharides and the Azure A cationic dye. Absorbance was then measured at 540nm. Standards of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL of fucoidan F5631 sulphated polysaccharide was used and data was recorded as percentage equivalents of fucoidan.

Silica-gel thin-layer chromatography (TLC) was used as a qualitative determinant of the monosaccharide content of PPE following the methods of Popper and Fry (Popper and Fry, 2003). 10mg of PPE was dissolved in 1 ml of 2 M trifluoroacetic acid and heated at 120°C for 1 hour to achieve hydrolysis, then centrifuged at 2000g for 5 mins. 5µL of PPE, and monosaccharide standards consisting of fucose, mannose, xylose, glucose, galactose, rhamnose, and arabinose, were added 0.8cm apart on the Silica Gel 60 plate stationary phase and allowed to dry. Plates were then inserted into a TLC development tank and ran in 500mL of mobile phase

consisting of dH_2O: butanol: acetic acid (1:3:1, v/v) for 8 hrs. The TLC plate was removed and allowed to dry at room temperature. The TLC plate was then stained using thymol-sulfuric acid staining (0.5g thymol: 95ml ethanol: 5ml sulphuric acid) and left to dry at room temperature before incubation at 70°C for 1 hour.

Structural information of each seaweed extract was determined using Fourier Transform Infrared Spectroscopy (FTIR) using the Bruker Tensor 27 FT-IR spectrophotometer (Bruker Corporation, UK). 1-2mg of seaweed extract powder, before and after *in vitro* digestion, was pressed into a disk and the IR spectrum was analysed with a scan range of 4000–850 cm^{-1}. Two hundred scans were taken with 4 cm^{-1} resolution with an aperture of 3mm. Background reads were taken before the sample. Atmospheric manipulation was incorporated, and vector normalisation was complete between 1700cm^{-1} and 900cm^{-1} using OPUS 5.5 software.

8.4 Mineral and trace metal content of whole seaweeds and seaweed extracts

Mineral and trace metal analysis of 1 x 10g of each whole seaweed and seaweed extract was outsourced to Advanced Laboratories (USA). Concentrations of aluminium, copper, sodium and zinc were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Concentrations of mercury, lead, arsenic, and cadmium were determined by inductively coupled plasma mass spectrometry (ICP-MS). Iodine concentration was determined using AOAC 932.21.

8.5 *In vitro* digestion

An *in vitro* simulated digestion was completed on the whole seaweed powders, polysaccharide-rich extracts, and purified seaweed polysaccharides, following the

method of Minekus *et al.* (Minekus et al., 2014), with oral, gastric, and intestinal phases of digestion. All simulated fluids were incubated at 37°C before use. Oral phase: 30g of whole seaweed powder or polysaccharide-rich extract was suspended in 21ml of Simulated Salivary Fluid (SSF), pH 7.0 (15.1mM KCl; 3.7mM KH_2PO_4; 13.6mM $NaHCO_3$; 0.15mM $MgCl_2(H_2O)_6$; 0.06mM $(NH_4)_2CO_3$). 150µL of 0.15M calcium chloride stock and 5.85ml H_2O were added and the warmed to 37°C. 3ml of 750 U/ml human salivary α-amylase stock solution was warmed to 37°C and added to the product suspension to achieve a final concentration of 75 U/ml. The suspension was incubated for 2 mins at 37°C in an orbital shaker (160 rpm). Gastric phase: 22.5ml of Simulated Gastric Fluid (SGF) (6.9mM KCl; 0.9mM KH_2PO_4; 25mM $NaHCO_3$; 47.2mM NaCl; 0.1mM $MgCl_2(H_2O)_6$; 0.5mM $(NH_4)_2CO_3$ and adjusted to pH 3.0 with 2M HCl) was added to the oral suspension, followed by 2.085ml of H_2O, 0.6ml of 1M HCl (to achieve a pH of 3) and 15µL of 0.15M calcium chloride. 4.8ml of a 25000 U/ml porcine pepsin stock solution was warmed to 37°C and added to the suspension to achieve a final concentration of 2000 U/ml. The final gastric suspension was incubated in an orbital shaker (160 rpm) for 2 hrs at 37°C. 450µL of 1M NaOH was added to the gastric chyme suspension to obtain pH 7.0. 33ml of Simulated Intestinal Fluid (SIF) (6.8mM KCl ; 0.8mM KH_2PO_4; 85mM $NaHCO_3$; 38.4mM NaCl; 0.33mM $MgCl_2(H_2O)_6$ and adjusted to pH 7.0 with 2M HCl) was added, followed by 7.5ml of a 160mM porcine bile extract stock solution to achieve a final concentration of 10mM. 15ml of an 800 U/ml porcine pancreatin stock solution was added to achieve a final concentration of 100 U/ml. The final intestinal suspension was incubated in an orbital shaker (160 rpm) for 2 hrs at 37°C. The intestinal phase digesta was dialysed for 24 hrs using 1kDa dialysis tubing to mimic intestinal absorption (Spectrum Labs, USA) and the retentate was freeze

dried (Labconco, USA) to obtain powders prior to batch culture fermentation. The *in vitro* digestion of purified seaweed polysaccharides method was scaled down for 1g starting mass.

8.6 Batch culture fermentation experiments

8.6.1 Preparation of human faecal inoculums

A 20% faecal slurry was prepared following the methods of O'Donnell *et al.* (O'Donnell et al., 2016), where 400g faeces were collected from six healthy human subjects with no autoimmune or allergic diseases and no antibiotics for the previous 6 months. Samples were processed within four hrs of collection and placed into large stomacher bags in a dedicated anaerobic biological cabinet under nitrogen gas. 400mL of 50mM sterile phosphate buffered saline was added to the stomacher bag and the slurry was homogenized. The slurry was then centrifuged at 3696 RCF for 25 mins in a Sorvall SLA-3000 rotor and resuspended to 400ml in PBS. The resulting faecal bacteria suspension was amended with sterile glycerol to a final concentration of 25% (v/v) and stored frozen at -80°C.

8.6.2 Preparation of basal media

Nutrient basal medium stock solution was prepared using methodology of Fooks and Gibson (Fooks and Gibson, 2003). Reagents (mg/L): Tryptone Water = 2,000; Yeast Extract = 2,000; Cysteine HCl = 1,000; Bile Salts = 500; Candidate carbohydrate = 10,000; Tween 80 = 2mL; Hemin = 50 (dissolved in 3 drops of 1M NaOH); Vitamin K_1 = 10µL; Antifoam = 200µL; NaCl = 100; KH_2PO_4 = 40; K_2HPO_4 = 40; $CaCl_2.6H_2O$ = 40; $MgSO_4.7H_2O$ = 10; and $NaHCO_3$ = 2,000. The media was sterilised using an autoclave (121°C for 15 mins) after production.

8.6.4 in *vitro* batch culture fermentation: individual seaweed polysaccharides

Digested, freeze-dried, seaweed polysaccharide powders underwent *in vitro* batch culture faecal fermentation using the MicroMatrix bioreactor with a 24-square well cassette (Applikon Biotechnology, The Netherlands) (O'Donnell et al., 2018). Synergy 1 (Beneo, Germany) was used as a positive control (n=8) and cellulose was used as a negative control (n=8). Seaweed polysaccharides were fermented in quadruplicate. Cassette preparation and sampling took place in an anaerobic cabinet under N_2 gas. Slurry was thawed at 37°C in an anaerobic cabinet prior to inoculation. Well preparation: 6.65ml media was added to each well of the cassette and faecal slurry was seeded at 5% (v/v). 2ml was removed from each well to provide a 0 hrs sample, before adding a carbon source at 1% (w/v) to the final 5ml volume. The cassette was sealed within the anaerobic cabinet and transferred to the MicroMatrix bioreactor workstation. The MicroMatrix bioreactor was operated using MicroMatrix Human Machine Interface software (Applikon Biotechnology, The Netherlands), with the following parameters measured and controlled throughout: orbiter speed (250 rpm), pH 6.8 (liquid addition of 4M NaOH), temperature (37°C), dissolved oxygen control (individually controlled gas addition of N_2 and CO_2). The bioreactor was switched off after 24 hrs of fermentation and the cassette was transferred to an anaerobic cabinet to take the 24 hrs samples. Samples were immediately centrifuged at 21,382 RCF to provide samples for DNA extraction (pellet) and SCFA analysis (supernatant). All samples were stored at -80°C until analysis.

8.6.6 *in vitro* batch culture fermentation: whole seaweeds and seaweed extracts

In vitro batch culture fermentation was performed using the Multifors parallel bioreactor with Iris 6.0 software (Infors HT, Switzerland). 2g of either FVWS, FVE, LDWS, LDE, PPWS, PPE, Cellulose (negative control), or Synergy 1 (positive control) was added to 190 ml nutrient basal medium stock solution (final carbohydrate concentration = 1% w/v). Vessels were sparged with N_2 gas for 2 hrs (2 psi) before inoculation with 10ml of 20% faecal slurry (previously thawed at 37°C). Vessels were stirred at 200 revolutions min^{-1}, maintained at pH 6.8 and 37°C. Samples were taken at 0, 5, 10, 24, 36 and 48 hrs. Samples were immediately centrifuged at 21,382 RCF to provide samples for DNA extraction (pellet) and SCFA analysis (supernatant). All samples were stored at -80°C until analysis.stored at-80°C for future analysis. During the *Palmaria palmata* red seaweed experiment, 100 µL fermentation media was taken for bacterial culture before centrifugation.

8.7 Culture-dependent analysis

Culture-dependent analysis was performed for the whole seaweed and seaweed extract fermentation of *Palmaria palmata* only. 100 µL of fermentation vessel sample was serially diluted in maximum recovery diluent (MRD) (10^1 - 10^5) and dilutions were plated in triplicate on MRS Agar selective for *Bifidobacterium* (sterile filtered with 5ml/100ml Mupirocin and 1ml/100ml Nystatin) and LBS Agar selective for *Lactobacillus* (sterile filtered with 1ml/100ml Nystatin). Cultures were incubated at 37°C for 72 hrs before counting. LBS plates were incubated in anaerobic jars with Anaerocult® A (Merck Millipore, USA) gas packs in a 37°C incubator. MRS plates were incubated for 72 hrs at 37°C in an anaerobic biological

cabinet under N_2 gas. Counting was performed using the Stuart Digital Colony Counter (Cole-Parmer, UK). Counts were corrected for each dilution factor and then calculated as $\log_{10}$ CFU/mL. Statistical analysis was performed using IBM SPSS Statistics (version 24) and GraphPad Prism 8 software. Graphs were made in GraphPad Prism 8 software. Statistical significance was determined using a non-parametric Kruskal-Wallis test (*p* value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (*q* value) and a significance level of 0.05.

8.8 DNA extraction

Genomic DNA was extracted from fermentation vessel media using the PowerFecal DNA extraction kit (Mo Bio Laboratories, Carlsbad, USA) according to manufactures' instructions. The bead beating step was completed using the Mo Bio vortex adapter. For the whole seaweed and seaweed extract fermentation experiments, DNA was extracted from samples obtained at 0, 5, 10, 24, 36, and 48 hrs fermentation. For the purified seaweed polysaccharide fermentation experiment, DNA was extracted from samples at 0 and 24 hrs fermentation.

8.9 Quantitative PCR

Total Bacteria, *Lactobacillus*, and *Bifidobacterium* were quantified using qPCR. Standards of 10^{10} CFU/ml of Total Bacteria, *Lactobacillus*, and *Bifidobacterium* were kindly provided by Dr. Michelle O'Donnell (Teagasc Food Research Centre Moorepark, Ireland). Standards were originally generated via DNA extraction from *E. coli* for total bacteria; from *Lactobacillus ruminis* for *Lactobacillus* spp.; and from *Bifidobacterium lactis Bb12* for *Bifidobacterium* spp., followed by amplification of the 16S target region and cloning of each fragment into E.coli TOP 10 chemically

competent cells using the TOPO TA Cloning Kit according to the manufacturer's instructions (Thermo Scientific, USA). Plasmid extraction was completed using the QIAprepSpin Miniprep Kit (Qiagen, USA) following the manufacturer's protocol. The obtained plasmid DNA was then digested using the Xbal restriction enzymes and restriction digest products were purified using the QIAquick PCR Purification Kit (Qiagen, USA). Dilutions of the linearised plasmid (1:5, 1:10, and 1:20) were then quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA) and used to calculate the molecular weights of PCR products. Copies of the linearised plasmids were calculated as copies/µl and working stocks of each standard were prepared with $1x10^{10}$ copies/µl.

The below calculation was then applied to generate the gene copy numbers for standards. Colony forming units (CFU) were calculated from the copy number results from each qPCR reaction using the following formula:

$$[(C/\mu l)(TV) \times (T\ cfu/ml)]/TCN = [cfu/ml(S)]/1.$$

C/µl = Copy number/µl

TV = Template volume

TC = Total copy number of the standard used

T cfu/ml = Total cfu/ml of standard used

cfu/ml(S) = cfu/ml of test sample

Primer sequences used for qPCR (Target: Forward primer 5'-3'; Reverse primer 5'-3'; Size bp; Tm °C): Total Bacteria (Eubacterial): ACTCCTACGGGAGGCAGCAG; ATTACCGCGGCTGCTGG; 200 bp; 60°C. Lactobacillus genus: GCAGCAGTAGGGAATCTTCCA; GCATTYCACCGCTACACATG; 349 bp; 60°C.

Bifidobacterium genus: CTCCTGGAAACGGGTGGT; GCTGCCTCCCGTAGGAGT; 203 bp; 60°C.

A standard curve of 10^9-10^3 CFU/ml was prepared in duplicate for each plate. The following PCR master mix was prepared: Forward primer = 0.1µl/sample; reverse primer = 0.1µl/sample; SYBR® FAST pPCR Master Mix (KAPA Biosystems, USA) = 5µl/sample; PCR water = 3.8µl/sample. 1 µl of sample DNA was added to 9 µl mastermix per well (reaction volume = 10 µl). The negative control was prepared using 1 µl PCR water. Each sample was run in duplicate on two plates (n=4). Plates were sealed and centrifuged at 1000 RCF for 30 seconds and loaded on to Lightcycler® 480 Instrument II (Roche, Switzerland). The PCR conditions were denaturation = 1 cycle; amplification = 40 cycles; melting = 1 cycle; cooling = 1 cycle. Target temperature was 95°C with a hold time of 3 min and a ramp rate of 4.4°C/sec. Statistical analysis was performed using IBM SPSS Statistics (version 24) and GraphPad Prism 8 software. Graphs were made in GraphPad Prism 8 software. Statistical significance was determined using a non-parametric Kruskal-Wallis test (*p* value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (*q* value) and a significance level of $q \leq 0.05$.

8.10 16S rRNA amplification and MiSeq sequencing

Illumina MiSeq sequencing library preparation was completed following the 16S metagenomic sequencing library protocol (Illumina, USA) and Fouhy *et al.* (Fouhy et al., 2015). Amplicon PCR: Genomic DNA was amplified using primers specific to the V3-V4 hypervariable region of the 16S ribosomal RNA gene to create a 460bp amplicon. These primers also incorporated the Illumina overhang adaptor

(Forward primer 5’

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

reverse primer 5’

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Each PCR reaction contained 2.5 µL template DNA, 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 12.5 µL 2X Kapa HiFi Hotstart ready mix (KAPA Biosystems, USA) - 25µL final reaction volume. PCR amplification was carried out using the Applied Biosystems 2720 thermal cycler (Life Technologies, USA) with the following parameters: heated lid 110°, 95°C for 3 mins; then 25 cycles of: 95°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs; hold at 72°C for 5 mins; hold at 4°C. A 3 µl sample of each PCR reaction was mixed with 5 µl of 6x DNA loading buffer (Bioline, UK), and loaded onto a 1.5% agarose gel (1X TAE buffer) with 2 µl of Midori green added. 5 µl of 100bp hyperladder (Bioline, UK) was used to check DNA bands. Gel electrophoresis was run for 30 mins at 100V and visualised with a UV filter.

Successful PCR products were removed of free dNTPs, salts, Taq and primers PCR products using Agentcourt AMPure XP kit (Beckman Coulter Genomics, UK). Using a multichannel pipette, 20 µl of homogenised room temperature XP bead solution was added to each well and pipetted up and down 20 times to ensure complete mixing. The samples were then incubated at room temperature for 5 mins before being placed on a 96 well magnetic plate stand (Life technologies, Carlsbad, USA) for 2 mins to allow the supernatant to clear. Using a multichannel pipette, the supernatant was carefully aspirated out and discarded. A series of 2 washes was carried out by adding, incubating for 30 seconds, and then removing 200 µl of 80%

ethanol. The plate was air dried at room temperature for 10 mins. The PCR plate was then removed from the magnetic stand. Using a multichannel pipette, the beads were re-suspended in 52.5 µl of 10mM Tris (pH 8.5). The PCR plate was placed back onto the magnetic stand for 2 mins and 50 µl of the cleaned sample was transferred into a new PCR plate.

A second PCR reaction was completed on 5 µL of the purified DNA to attach dual indices and Illumina sequencing adapters onto the amplicons, using the Nextera XT Index kit (Illumina, USA). Each PCR reaction contained 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix, and 10 µl PCR grade water. PCR conditions were as described above, with only 8 amplification cycles. PCR products were visualised and cleaned as described above.

The DNA concentration of each sample was determined using the Qubit High Sensitivity DNA kit and the Qubit 3 Fluorometer (Invitrogen, USA). DNA samples were then pooled to an equimolar mix following Illumina guidelines. A final clean-up was conducted on the equimolar mix using 600 µl (1:1) AMPure XP bead solution (Beckman Coulter Genomics, UK) and incubated at room temperature for 5 mins and placed on a magnetic stand for 10 mins. The supernatant was aspirated off and discarded. The bound beads were washed twice by adding 200 µl of 80% ethanol. Ethanol was removed by aspiration after incubated for 30 seconds and the tubes were air dried at room temperature for 10 mins. The tube was removed from the magnetic stand and the beads were re-suspended in 25 µl of 10mM Tris pH 8.5. The tube was placed back on the magnetic stand and incubated for 5 mins. 25 µl of the supernatant was removed and transferred to a new 1.5ml microtube and stored at -20°C until pre-sequencing steps were carried out.

The Agilent Bioanalyser (Agilent Technologies, USA) was used for quality control before sequencing, then samples were sequenced on the MiSeq sequencing platform (Teagasc Food Research Centre Moorepark, Cork, Ireland), using a 2 x 250 cycle kit, following standard Illumina sequencing protocols.

8.11 Bioinformatics and statistical analysis

Two hundred and fifty base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (Caporaso et al., 2010). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123.

Statistical analysis of 16S rRNA amplicon sequencing data was carried out using Calypso online software (version 8.68) (Zakrzewski et al., 2017). Data were normalized using cumulative sum scaling and $\log_2$ transformed to account for the non-normal distribution of sequencing data (Paulson et al., 2013). Up to 20,000 taxa with > 0.01% abundance were used in the analysis. Chloroplasts and cyanobacteria were removed from the analysis.

Alpha diversity was determined using rarefied Chao1, Evenness, Shannon and Simpson indices. Statistical significance was determined using a non-parametric

Kruskal-Wallis test (p-value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value). Beta diversity was determined Bray-Curtis dissimilarity distance matrices for each fermentation substrate and cellulose at a given time point. A Permutational multivariate analysis of variance (PerMANOVA) was used to determine the statistical difference between Bray–Curtis dissimilarity indices of beta diversity.

Statistical significance of mean bacterial relative abundances, compared to cellulose, at the phylum, family, and genus level after 0, 10, and 24 hrs fermentation was determined using a one-way ANOVA with a Tukey's post-hoc test for multiple comparisons and a significance level of $p \leq 0.05$. Discriminate taxa between fermentation substrates and cellulose at a given time point were identified using linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011).

8.12 Short chain fatty acid and branched chain fatty acid analysis

The concentration of short chain fatty acids and branched chain fatty acids in fermentation vessel media supernatants was analysed using gas chromatography-mass spectrometry (GC-MS) using a modified protocol described by Garcia-Villalba *et al.* (Garcia-Villalba et al., 2012). Phosphoric acid was added to samples to a final concentration of 0.5% (v/v) and vortexed to homogenise for 30 seconds. Samples were then centrifuged at 21,382 RCF for 10 mins and the supernatant was filtered through 0.2µm filters. 1ml of ethyl acetate was added to 1ml filtered supernatant (1:1 ratio of extractant solvent to sample matrix), vortexed to homogenise, and a minimum period of 2 mins was used for extraction (until clear phase separation was observed). Samples were centrifuged at 21,382 RCF for 10 mins, and 600µl of

the organic phase (upper layer) was removed. 180µl of the organic phase was added to a GC vial, alongside 20µl internal standard (4-methyl valerate). A standard curve of 10, 20, 50, 100, 500, 1000, 5000, 10000, 50000, and 100,000 µM SCFA mix, containing acetic acid, propionic acid, n-butyric acid, i-butyric acid, valeric acid, i-valeric acid, and hexanoic acid was ran within every sample batch. Ethyl acetate blanks were run after every six vials, and in between each standard vial/QC to prevent carryover.

The GC-MS system consisted of an Agilent 6890N (Agilent Technologies, USA), equipped with an Agilent 7683 AutoSampler and 7683B injector, coupled to an Agilent 5973 inert mass selective detector. Agilent MassHunter GC/MS Acquisition software was used. The GC was fitted with a DB-WAXetr capillary column (30m length, 0.25mm i.d, 0.25µm film thickness), with helium used as the carrier gas (1.2mL/min). Injections were made in splitless mode with an injection volume of 1µL (10 µL syringe) and an injection temperature of 250°C. The syringe undertook four pre-washes and four post-washes in hexane. Sample skim depth was 3mm and four sample pre-washes and five sample pumps were completed. A glass liner with a glass wool plug at the end of the liner was used to avoid contamination of the GC column with non-volatile materials from faecal fermentations. An ethyl acetate blank was run every 6 samples. An autotune and tune evaluation was completed to check the amount of H_2O, N_2, O_2, CO_2 in the system. The initial column temperature was 90°C, then increased to 150°C at 15°C/min, then to 170°C at 5°C/min, then to 230°C at 20°C/min, which was maintained for 2 mins. Total run time was 14 mins. Solvent delay was 2.5 mins. The detector was operated in electron impact ionisation mode. Statistical analysis was performed using IBM SPSS

Statistics (version 24). A one-way ANOVA with a Tukey's post-hoc test to adjust for multiple comparisons was used with a significance level of $p \leq 0.05$ for whole seaweed and seaweed extract experiments. A non-parametric Kruskal-Wallis test (p value) with a Benjamini and Hochberg false discovery rate (FDR) correction for multiple comparisons (q value) was used for the individual polysaccharide experiment with a significance level of $q \leq 0.05$.

8.13 References

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9 Appendix 2 – CPD Activities

9.1 Conferences

- Irish Plant Scientists' Association 11th – 12th May 2015 (oral)
- NutraMara Conference, 29th – 30th June 2015 (poster)
- The Nutrition Society Spring Meeting, 21st – 22nd March 2016 (poster)
- ISAPP Students and Fellows Association, 27th – 29th June 2017 (poster)
- The Nutrition Society Student Conference, 7th – 8th September 2017 (oral)

9.2 Voluntary

- Student Representative, The British Phycological Society, 2015 – 2017
- Early Career Symposium Committee, The Physiological Society, 2016
- Treasurer/Secretary, ISAPP Students and Fellows Association, 2016 – 2018